New Series Vol. XVI No. 64 October 1952

Price 20*s*. net

ANNALS OF BOTANY

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OXFORD : AT THE CLARENDON PRESS

LONDON : GEOFFREY CUMBERLEGE

PRINTED IN GREAT BRITAIN
BY CHARLES BATEY AT THE UNIVERSITY PRESS, OXFORD

1952

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The Journal is published four times yearly by Geoffrey Cumberlege, Oxford University Press, Amen House, Warwick Square, London, E.C. 4, to whom subscriptions and all communications, other than editorial, should be addressed.

The subscription price for four numbers is 50*s.* (foreign postage, 1*s.* 6*d.* extra), single copy 20*s.* net. Cloth cases may be obtained, price 7*s.* 6*d.*

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Ascospore Dimorphism and Mating Type in *Chromocrea spinulosa* (Fuckel) Petch n. comb.

BY

M. J. MATHIESON[1]

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With Plates XXI and XXII and two Figures in the Text

Abstract

The asci contain four large and four small ascospores, each two-celled, arranged in the six patterns expected if spore size were controlled by a pair of allelic genes, the locus showing 65 per cent. second-division segregation. The small ascospores produce sterile colonies, the large ones moderately fertile colonies whose asci again show segregation for spore size. Fertility is stimulated where colonies from large and small spores meet. In crosses of various mutants with the wild type, all asci in perithecia developed along the line of junction show segregation for the mutant as well as for spore size. Evidently *Chromocrea* is heterothallic, the spore-size difference being a pleiotropic expression of mating type. One allele, that governing large spores, occasionally mutates to the other allele, resulting in fertility of colonies from large spores.

Introduction

Chromocrea spinulosa (Brooks and Mathieson, 1950) is assigned to the family Hypocreaceae as the perithecia are light coloured and develop in cushion-like stromata. There are no asexual spores. The asci are cylindrical and contain initially eight spores each of which subsequently divides into two so that at maturity there are sixteen free spores in each ascus. The ascospores contain a green pigment which is also present in the cells round the ostioles of the perithecia. This character is apparently sufficient to transfer the fungus from the genus *Hypocrea* to the genus *Chromocrea*.

The two cells of the eight spores are slightly unequal in size and shape, the upper cell being approximately cubical and the lower cell obovate. In addition to this there is a size difference amongst the two-celled spores, four being larger than the remaining four. The larger two-celled spores are 6·5–9 μ in length and 3 μ in width, the smaller two-celled spores are 5–6 μ in length and 2·5–3 μ in width. This character segregates in the ascus as if it was controlled by a pair of allelomorphs. When the two types of ascospore were isolated and germinated, the larger spores gave rise to colonies which developed stromata and perithecia while the smaller spores gave rise to colonies which remained completely sterile. Not only did the larger spore colonies

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[Annals of Botany, N.S. Vol. XVI, No. 64, October, 1952.]

produce perithecia but the asci again showed segregation for spore size and when germinated gave rise to the two types of colony described above.

This paper deals with the relation between the spore dimorphism and the mating behaviour of the fungus, and with the evidence for regarding the fungus as heterothallic.

Methods

The wild type stock used in this work was a direct subculture from ascospores of the original isolation by Dr. E. G. Bollard. This isolation was made from dead culms of *Dactylis glomerata* at Cambridge in December 1945.

Dissection of asci. Asci were dissected with a *de Fonbrune* micro-manipulator. Clusters of asci were mounted in a drop of 2 per cent. malt extract in water on sterile $1 \times \frac{7}{8}$ in. coverslips. Ten to twelve drops of 2 per cent. malt extract agar were arranged on the coverslip on either side of the drop of malt extract and the whole coverslip was then inverted over a cell in which a moist atmosphere could be maintained and the dissecting tools inserted from the side. Spores were dissected from the asci on the glass and then transferred to the solidified drops of agar. Germination was usually complete within 12–14 hours at 25° C. and the agar drops with their germinated spores were then transferred to agar slopes. At first all sixteen spores were germinated separately, but later as each group of four was found to be genotypically the same, they were dissected and germinated in groups of four.

Media. Although growth was satisfactory on a chemically defined medium, i.e. the usual mineral salts with sodium nitrate as a source of nitrogen and glucose as a source of carbon, perithecial production was poor. Optimum growth and development of perithecia was obtained on 2 per cent. malt extract with a pH of approximately 5·6. This medium was used for the major portion of the work.

Growth rate. Linear growth rate was measured as mean increase in colony diameter over a period of 24 hours, when the colony was grown in a 9-cm. Petri dish containing 20 c.c. of medium.

Ultra-violet irradiation. Mature ascospores were suspended in sterile water at a concentration of approximately 10^5 per c.c. The suspension was filtered through cotton-wool to remove clumps of spores and hyphal debris and the filtrate irradiated inside a quartz tube. The tube was fixed parallel to a mercury vapour lamp and could be rotated throughout the exposure so that the amount of shadowing was approximately equal for all spores. The approximate value of the radiation at the position of the tube was 10,000–12,000 ergs/cm.^{2}/sec. After irradiation dilutions were made in sterile water and plated in malt agar.

X-radiation. A dense suspension of ascospores was spread on the surface of sterile malt agar in a watch-glass and covered with cellophane. The watch-glass was placed 1 cm. from the aluminium filter of the machine which was approximately 20 cm. from the source. The machine had an output of 750 r./minute, and the spores were given a total dose of 45,000 r. After treat-

ment the spores were washed off the surface of the agar with sterile water and dilutions made on malt agar plates.

Cytology of the ascus. Young stromata were fixed in 1:3 acetic alcohol for 24 hours. Before staining it was found necessary to mordant in 4 per cent. iron alum for ½ hour (Fincham, 1948). The stromata were then washed in distilled water and transferred to aceto-carmine. The asci were removed from the perithecia, warmed slightly to spread under the coverslip, and then squashed.

Stromata were also embedded in paraffin and sectioned. The sections were stained according to Newton's gentian-violet method.

Results

Description of wild type cultures. Visible growth appeared on a malt agar plate from a mass inoculum of ascospores after 24 hours at 25° C. The mycelium was colourless and spread evenly over the surface of the agar at first, but aerial mycelium quickly developed and after reaching the lid of the dish spread radially over the surface of the glass. After a colony about 3–3·5 cm. in diameter had developed, the type of growth changed from an even regular radial growth where all the hyphae appeared to grow at the same rate, to one where some parts of the mycelium had a higher growth rate than others, so that numerous fan-like sectors were produced. In these sectors stromata started to develop, the concentration of stromata varying throughout the colony. The growth of the sectors was not sufficiently well defined for any pattern of stromatal development to be made out. The linear growth rate varied from 12 to 15 mm. in 24 hours at 25° C. Growth of the stromata continued after mycelial growth had ceased, and ascospores were always mature after 12 days. All asci showed segregation for spore size. In the mature colony stromata could be found in all stages of development, some with no perithecia at all, some with very few, and others with perithecia fully developed. The number of fertile stromata in wild type colonies ranged from 250 to 300.

The optimum temperature for growth and development of stromata was 25° C. Growth was zero at 30° C. Within the range 18–27° C. the number of stromata produced was not affected by temperature, but the development of perithecia was retarded below 20° C. and at 18° C. very few perithecia matured.

Single ascospore cultures. The convention employed in describing these different strains will be as follows: single large spore colonies will be referred to as wild type (l^+) and single small spore colonies as the mutant type (l). A colony derived from a mixed mass inoculum of these two types of spore will be described as mixed wild type (l^+ l).

(*a*) The large (l^+) spore type: The growth rate was approximately the same as that of a colony from a mixed inoculum, 10–13 mm. in 24 hours, but the numbers of stromata formed was considerably less than in a mixed wild

type colony, and probably on this account the stromata were larger and contained more perithecia (Fig. 2). The asci still showed segregation for spore size. Single l^+ spores were grown on 2 per cent. malt agar at 18°, 22°, and 25° C. and the number of stromata counted. The number ranged from 45 to 80 per plate in all cases and was not affected significantly by temperature. The number of stromata which produced perithecia, however, increased with the temperature. Variation in the number of l^+ spores in the inoculum from 1 to 16 had no effect in the type of colony or number of stromata produced.

(*b*) The small (*l*) spore type: This was identical with the l^+ spore type except that no stromata were formed nor perithecia developed, i.e. the colony was completely sterile.

Mixed cultures of the l^+ and l spore types. Single l^+ and l spores were inoculated on opposite sides of a malt plate. No stromata were formed in the colony from the l spore and the l^+ colony formed the usual scattered stromata, but at the junction of the two colonies a line of stromata and perithecia was developed. When two l^+ spores were grown in this way no such line was developed, the stromata remaining scattered at random throughout both colonies.

This would suggest that even though l type mycelium is not essential for the production of perithecia, it does have a stimulatory effect on their development in colonies of the l^+ type. This stimulation could be merely nutritive. If this were the case, then it must be caused by some unique property of the l type mycelium itself and not by depletion of food material, as a similar effect is not produced when two l^+ colonies are grown together in this way. The other alternative is that there is a sexual fusion between the two strains followed by an increased development of perithecia. It was impossible to demonstrate the hybrid nature of perithecia when the only difference between the two strains was spore size and colony character, since both of these segregate in single l^+ cultures. Therefore spores were irradiated in an attempt to incorporate additional mutant characters in the stocks.

Isolation of mutants. Colonies which were obviously different from wild type were isolated from the dilution plates after irradiation with ultra-violet light. The majority of these differed from wild type in having a lower growth rate, a different type of growth, and furthermore they were sterile. Five of these mutants were selected for further work. An additional 265 colonies from spores treated with ultra-violet light and 350 from X-rayed spores were grown to maturity and examined for spore-colour mutants. Two spore-colour mutants (strains 1 and 2) were obtained from the self-fertile survivors after X-ray treatment. In both of these the stromata were pure white instead of buff-coloured, and the spores were colourless. They still showed segregation for spore size and the l colony types were extracted from both. These were phenotypically indistinguishable from l wild type. Four additional albino strains (4, 5, 6, and 7), were isolated from the X-ray treated spores,

and these were all of the *l* type. A mutant (strain 3) with pale green ascospores was also obtained. Tests for allelism showed that the six albinos fall into at least two groups, strains 1 and 2 being allelic and strains 4–7 being non-allelic with 1 and 2. Since the albino spores of strains 4–7 failed to germinate, they could not be separated further. The growth rate of the albino colonies was the same as that of wild type.

Description of mutants. The following five morphological mutants were used in crosses with wild type.

1. Brown exudate (*brx*): The original isolation was apparently from an l^+ spore as the mutant developed perithecia. It differed from wild type in the production of a brownish exudate from hyphae at the apex of a slope and from the aerial hyphae in a plate culture. The growth rate was practically the same as wild type and cultures needed to be full grown before they could be distinguished with certainty. The l^+ and *l* spore colonies both possessed the mutant character and differed from each other as did their wild type counterparts. In colonies of the *brx* l^+ type fewer stromata were produced than in + l^{+*} colonies. (Pl. XXII, Fig. 4 G).
2. Fluffy (*fl*): Characterized by a fluffy aerial mycelium and reduced growth rate, about 2·5 mm. in 24 hours instead of 10–13 mm. as in wild type. At first the circumference of the colony was regular, but later became very uneven. The original isolation was sterile, which suggested it may have come from an *l* spore. (Pl. XXII, Fig. 4 H).
3. Irregular (*ir*): Aerial mycelium reduced and habit of growth very irregular and variable in density. Growth rate about 6 mm. per 24 hours. The original isolate was quite sterile. (Pl. XXII, Fig. 4 A, B.)
4. Dense (*den*): Very dense restricted growth with fluffy aerial mycelium. Growth rate approximately 0·5 mm. in 24 hours. The colony was quite sterile. (Pl. XXII, Fig. 4 E, F.)
5. Restricted (*re*): Regular but restricted growth, aerial mycelium reduced. Growth rate 2·0 mm. in 24 hours. The surface of the colony was densely covered with sterile stromata. (Pl. XXII, Fig. 4 C, D.)

Crosses between mutants and wild type. Only *brown exudate* and possibly *restricted* were of the l^+ type. The reaction of these two mutants in crosses with wild type was used therefore as a standard of reference for the reaction of the remaining mutants. The results are given in Table I; 's' indicates the presence of stromata.

From all those plates in which there appeared to be a stimulation of perithecial production the asci contained in these perithecia were found to be hybrid, indicating that they were formed after the fusion of mutant and wild

* The usual genetic symbols are employed in this paper, namely, lower-case italic letters for the mutant gene and the same letter combination with a superscript plus sign for its normal or wild type allele, thus *brx* and brx^+, *l* and l^+. In some cases, as here and in Table I, the wild allele is represented by a plus sign alone where the context allows no ambiguity as to which wild type gene is intended.

type nuclei. All asci in perithecia formed in $+ l^+$ colonies were wild type and hybrid only for spore size, similarly perithecia formed within the *brx* colony were *brx* and hybrid only for spore size. In four of the five mutants hybrid perithecia were formed at the junction of mutant and wild type colonies when the suitable cross was made, but in the fifth, *dense* × wild type, hybrid perithecia were formed in the $+ l$ colony only, the mutant colony being very small and surrounded by wild type.

TABLE I

Mutant.	Wild type.	Region of production of stromata: Junction of mutant and wild type.	Region of production of stromata: Within wild type colony.	Region of production of stromata: Within mutant colony.	Inferred spore type of mutant.
brx	$+ l^+$	—	s	s	l^+
brx	$+ l$	s	—	s	
fl	$+ l^+$	—	s	—	l^+
fl	$+ l$	s	s	—	
ir	$+ l^+$	s	s	—	l
ir	$+ l$	—	—	—	
den	$+ l^+$	—	s	—	l^+?
den	$+ l$	—	s	—	
re	$+ l^+$	—	s	—	l^+
re	$+ l$	s	s	s	

From hybrid asci the four expected colony types were recovered, i.e. mutant l and l^+ and wild type l and l^+. The two mutant types were distinguishable in *brx* and *fl* in the same way as wild type by the fertility of the l^+ type and the sterility of the l type, and in *re* by the presence of sterile stromata in the *re* l^+ type and absence of stromata in the *re* l type. In both *ir* and *den* the two types can be distinguished only by their reactions when crossed with $+ l$, since they are phenotypically the same.

The analysis of hybrid asci showed that *ir*, *den*, and *re* segregate independently of the genes determining spore size. However, there was clear evidence of linkage between *brx*, l^+, and *fl*, the loci perhaps being in that order with the centromere to the left of *brx*. Recombination percentages were for *brx* l^+ 23 $\pm$ 4·1 based on 37 asci, for l^+ *fl* 16·2 $\pm$ 3·8 based on 37 asci, and for *brx fl* 25 $\pm$ 4·6 based on 30 asci.

These results suggest that *Chromocrea* is indeed heterothallic, and that the heterothallism is controlled by a single pair of alleles. Remarkably the mating type alleles are pleiotropic, leading to an ascospore size difference as well as a mating type difference. The behaviour of the l^+ spore colonies could be explained by postulating a mutation in mating type which occurs in colonies from spores of this type.

Heterocaryosis. If it is assumed that the fertility of the l^+ cultures is the result of a mutation in mating type, the behaviour of the mutant nucleus may be of two kinds: either it may fuse immediately with one of opposite mating

type and give rise to one or several perithecia, or it may continue to divide either independently of, or conjugately with, nuclei of the opposite mating type forming a heterocaryon.

Since there are no asexual spores formed by *Chromocrea* the only possible way to detect such a heterocaryon is to isolate hyphal tips and by the character of the resulting colonies determine the mating types of the nuclei present in the tip. Pieces of agar approximately 2 mm. square were taken from a $+\ l^+$ colony at different distances from the point of inoculum. One piece was transferred to a slope as a mass inoculum and an adjacent piece was used for the isolation of hyphal tips. The results of this analysis are given in Table II.

TABLE II

Distance in cm. from point of inoculation.	No. of hyphal tips.	Type of colony from hyphal tips		Mass inoc. Type of colony.
		l^+	l	
1	12	12	0	l^+
2	8	8	0	l^+
3	15	15	0	l^+
4	10	10	0	l^+

Although analysis of many more hyphal tips would be necessary before it could be said that l nuclei were absent from the mycelium, at least they must be in a minority, and certainly do not divide conjugately with l^+ nuclei.

Sansome (1946) found that in *Neurospora crassa* heterocaryons were not normally formed between strains of opposite mating type although heterocaryosis was possible when the mating types were the same, while in *N. tetrasperma* where nuclei of both mating types are normally associated throughout the life-history heterocaryons were readily formed between unisexual strains of opposite mating type. If *Chromocrea* resembles *Neurospora* in this respect such a condition may operate against the free reproduction and movement of l nuclei and an l^+ mycelium. Experiments with combinations of mutants, however, showed that heterocaryosis under these conditions is unrestricted. Both mating types of mutants *restricted*, *dense*, and *irregular* were combined in the twelve possible ways. When the inocula were placed together in the centre of the dish the colony in all cases had the characters and growth rate of wild type. When the inocula were placed 1·5 cm. apart the mutants retained their individuality until they met, then sectors of wild type growth developed from the line of junction and quickly spread over the unoccupied part of the plate.

Migration of nuclei. Dowding and Buller (1940) have shown that in *Gelasinospora tetrasperma* nuclei of one mating type can migrate through the mycelium of the opposite mating type following hyphal fusions. They have also shown that the rate of movement of the migrating nuclei is about 4–5 mm. per hour, which is faster than the rate at which the hyphae grow.

Nuclei of *Chromocrea* behave in a similar fashion, although the actual rate

of movement has not been determined. In crosses of the mutant *den* l^+ with wild type + *l*, the wild type mycelium grew so much faster than the mutant that it quickly surrounded it and grew beyond it. Stromata, instead of developing round the boundary of the mutant colony, appeared in the wild type mycelium 1·5–2 cm. beyond the mutant after the surface of the plate was covered (Text-fig. 1). Then about 10 days later stromata appeared on the opposite side of the dish in the wild type mycelium. Hyphal fusions were observed between mutant and wild type hyphae. Pieces of mycelium were

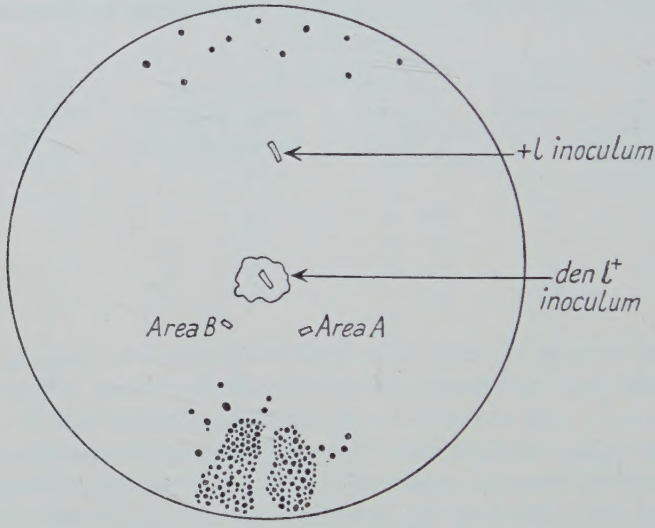

FIG. 1. Plan of distribution of stromata in a cross between wild type + *l* and the mutant *den* l^+.

removed from area *A* (Text-fig. 1) to hard agar and hyphal tips isolated. Each one produced a colony which was completely fertile and dissection of asci showed that they were all hybrid for the mutant and wild type. Hyphal tips isolated in the same way from area *B* gave the same result. Apparently, therefore, nuclei of one mating type are able to migrate freely through a mycelium containing nuclei of the opposite mating type. If the nuclei are able to migrate freely in either direction, i.e. from wild type to mutant and vice versa, then some factor is operating in the mutant colony to prevent the formation of perithecia. The same or some other factor also prevents formation of perithecia in the wild type colony except near the edge of the dish. Since hyphal tips from the wild type mycelium in areas *A* and *B* immediately give rise to stromata and perithecia when grown on fresh medium, the factor preventing their formation in the parent colony may be simply depletion of nutrients.

Further information about the movement of nuclei was obtained from crosses between *albino–1* and wild type. These mutants have the added

advantage of being readily detected in perithecia without dissection of asci. *Albino–1* (*al–1 l*$^+$) was crossed with + *l*. Pure albino stromata developed within the albino colony and a row of hybrid stromata appeared along the junction between the two. Wild type is apparently dominant to albino since the hybrid stromata were indistinguishable from wild type. Four days after inoculation immature stromata were transferred from the culture plates to hard agar and

Fig. 2. Plan of distribution of albino (open circles) and wild type (closed circles) stromata in the cross *al–1 l*$^+$ × + *l*. For numbers on stromata see Table III.

incubated overnight. Hyphal tips were then isolated to fresh tubes of agar. The stromata were removed from the plates before the ostioles had developed so that there was no risk of ascospores germinating and confusing the results of the hyphal tip analysis. A plan of the distribution of stromata is given in Text-fig. 2 and the results of the hyphal tip analysis in Table III. The phenotypically *l* colonies were tested against *fl l*$^+$ since they were otherwise indistinguishable from *al–1 l*. The colonies developing from hyphal tips were usually uniform, but three colonies from stroma 4 developed three different types of stromata within each colony, hybrid, pure albino, and mixed, i.e. part hybrid and part albino. These may have been simply the result of two individual stromata developing close together.

Two of the phenotypically hybrid stromata gave + *l* type colonies only. This may indicate that either + *l* nuclei were present in greater numbers than *albino* or that there were more present in the young hyphae developing from the stromata. The number of hyphal tips isolated may have been too small

to detect the *al–1 l*$^+$ nuclei, but 0:7 is not significantly different from 2:6. The remaining three hybrid stromata gave both hybrid and *al–1 l*$^+$ colonies. If the rates of division of the two types of nuclei were widely different, *al–1 l*$^+$ colonies would not be expected in the numbers obtained without $+$ *l* colonies also being present.

TABLE III

Phenotype of parent stroma.	Stroma number.	Colony type from isolated hyphal tips				
		al–1 l$^+$ $+$ *l*	$+$ *l*	*al–1 l*$^+$	*al–1 l*$^+$ *al–1 l*	Hybrid and *al–1 l*$^+$
Wild type from junction of colonies	2	2	6	—	—	—
	2*a*	9	—	6	—	—
	2*b*	—	7	—	—	—
	3	5	—	4	—	—
	4*a*	—	7	—	—	—
	Total	16	20	10	—	—
Albino	3*a*	—	—	2	6	—
	3*b*	10	—	2	—	—
	4	—	—	4	2	3
	4*b*	12	—	4	—	—
	Sub-total	22	—	12	8	3
Al–1 l$^+$ inoculum	—	8	—	2	—	—
	Total	30	—	14	8	3

There are two types of hybrid stromata, those with excess $+$ *l* nuclei and those with excess *al–1 l*$^+$ nuclei. The former are found only at the junction of the two colonies, the latter at the junction and in the albino stromata. This suggests that the former result from migration of *al–1 l*$^+$ nuclei and the latter from migration of $+$ *l* nuclei. If so, the stromata from wild type should rarely if ever give $+$ *l* colonies since presumably this would be a rare type of nucleus and all hyphal tip isolations from wild type stromata would give *l*$^+$ alone or *l*$^+$ *l*, and these two kinds would be phenotypically alike.

Only one of the albino stromata gave rise exclusively to albino colonies. All the rest were a mixture of hybrid and albino and yet all were phenotypically albino when selected for hyphal tip isolation. Of the eight hyphal tips from 3*a*, six produced colonies with such large numbers of stromata that they must have contained both *al–1 l*$^+$ and *al–1 l* nuclei. Similarly, from stroma 4, two of the six hyphal tip colonies were phenotypically *al–1 l*$^+$ and *al–1 l*. The only criterion in these cases was the relative numbers of stromata produced, which is only a qualitative difference and not absolutely conclusive.

Obviously $+$ *l* nuclei had migrated through *al–1 l*$^+$ hyphae and were present in 60 per cent. of the hyphal tips isolated. The stromata must have been initially albino since they developed before the two colonies met and they were isolated before the presence of the $+l$ nuclei was expressed phenotypically. Their presence was shown later in development when originally

albino stromata with albino perithecia and asci were observed to change to phenotypically wild type stromata by developing pigment round the ostioles. Some of the perithecia in such stromata were hybrid and some were pure albino. None of the perithecia examined from these crosses was mixed, i.e. contained asci which were hybrid as well as asci which were either all albino or all wild type.

There is no evidence from these results that nuclei which have arisen by mutation of mating type are free to migrate as independent units, because under these conditions one would expect at least some hyphal tips to be *al–1 l*. Presumably these are too rare to be isolated pure in a hyphal tip. The presence of such nuclei in conjunction with *al–1* l^+ nuclei, however, is indicated by colonies from stromata 3*a* and 4 which appear to have contained both *al–1* l^+ and *al–1 l* nuclei in the original hyphal tip.

If after a mutation in mating type either conjugate or independent division of the two nuclei took place, hyphal tips isolated from the periphery of such a colony should give rise to l^+ *l* type colonies as well as *l* and l^+. Nevertheless only l^+ have ever been isolated. If the *l* nuclei behave in the same way as they do when *l* and l^+ spores are grown together, l^+ spore colonies should contain sectors of l^+ *l* type arising from the point of mutation. No such sectors have been found.

The only alternative explanation is that the two nuclei associate close to the point of origin and give rise to a stroma. The number of mutations involved in the development of each stroma is not known, but it is unlikely that each mutation gives rise to a single perithecium since this would require a group of mutations close to each other in time and space.

Evidence has been obtained that there may be more than a single pair of nuclei involved in the development of the asci of a single perithecium. A cross was made between *al–1* l^+ and $+$ l^+ mycelia. If in each colony mutation to the opposite mating type occurs, followed by immediate association of the mutant and parent nuclei, then only albino or wild type asci would be formed and never hybrid asci. If, however, free $+$ *l* and *al–1 l* nuclei are present after mutation, the four types of nuclei $+$ l^+, $+$ *l*, *al–1* l^+, and *al–1 l* would be free to associate in pairs as follows: *al–1* $l^+ \times$ *al–1 l* giving albino asci, *al–1* $l^+ \times + l$ giving hybrid asci, $+ l^+ \times$ *al–1 l* giving hybrid asci, and $+ l^+ \times + l$ giving wild type asci. In the cross described above pure albino stromata were produced in the *al–1* l^+ colony and pure wild type in the $+$ l^+ colony. Where the two colonies adjoined the stromata were phenotypically wild type but all the perithecia contained hybrid asci. Some of the perithecia contained exclusively hybrid asci, others contained a mixture of hybrid and wild type asci; none was found with a mixture of hybrid and albino asci. There was no concentration of stromata along the line of junction between the two colonies, which suggests that there is no stimulation such as exists when strains of opposite mating type are crossed. Hyphal fusions between the two mycelia, followed by migration of nuclei and the formation of a heterocaryon, could give rise to such mixed perithecia. In

such a heterocaryon it is probably a matter of chance which pair of nuclei fuse provided they are of opposite mating type.

These observations would suggest that after mutation the mutant nucleus does not immediately associate with one of the opposite mating type although the association of the nuclei cannot be long delayed.

Development of the asci. Binucleate ascogenous hyphae and well-developed croziers are formed which show characteristic proliferation. The penultimate cell of the crozier elongates to form the ascus and the two nuclei fuse. At metaphase I of the fusion nucleus chromosomes are visible and there appear to be 8 bivalents, but no great reliance should be placed on this figure because of the smallness of the chromosomes. The spindles of the two meiotic divisions and the succeeding mitosis are placed longitudinally so that an ordered tetrad is formed. Spore walls are delimited round the nuclei in the 8-nucleate stage, another mitosis takes place, and a cross wall is formed between the two nuclei resulting in 16 uninucleate spores at maturity.

No obvious ascogonium was ever found and there are no spermatia or conidia of any kind. This would suggest that the mycelium at the base of the perithecium must become heterocaryotic for the two mating types by fusion of vegetative hyphae and migration of nuclei.

Segregation of spore characters. In *Chromocrea* the factor determining spore size is either the same factor which determines mating type or is so closely linked to it that no crossing over has occurred in 115 asci. If the fertility of the l^+ colonies is due to a mutation in mating type and if the factors for mating type and spore size are determined by separate closely linked loci it is unlikely but not impossible that the two mutations should always occur simultaneously. This argues in favour of regarding spore size and mating type as pleiotropic expressions of alleles of the same gene.

The relative frequencies of the six types of spore arrangement in the ascus were determined. The arrangements were classified as follows:

Class	1	2	3	4	5	6
	l^+	l	l^+	l	l	l^+
	l^+	l	l	l^+	l^+	l
	l	l^+	l	l^+	l	l^+
	l	l^+	l^+	l	l^+	l

A total of 2,161 asci were scored, and the following frequencies obtained.

TABLE IV

Sample.	Classes 1	2	3	4	5	6	Total asci.
1	112	93	78	92	70	78	523
2	82	81	86	86	89	80	504
3	73	98	103	108	96	78	556
4	84	83	67	67	67	69	437
5	24	24	27	19	23	24	141
Total	375	379	361	372	345	329	2,161

Obviously there is no significant deviation from equality between the two first division segregation classes. The two symmetric second division classes are slightly in excess of the asymmetric classes but the difference is not significant (χ^2 is 2·47 for 1 degree of freedom). The percentage of second division segregation is 65·1. Presumably therefore the locus governing spore size is so far from the centromere that it segregates independently of it.

Scoring the *albino–1* character in the ascus was more difficult since its presence seemed to prevent some of the asci from reaching maturity. It was only possible to score spore size as well as colour in a few cases because the albino spores in the ascus were usually so immature that the difference in size was not sufficiently distinct. In the cross *al–1* $l^+ \times +$ *l* the frequencies recorded in Table V were obtained. The classes are the same as those for spore size if the gene for green is substituted for l^+.

TABLE V

Sample.	Classes 1	2	3	4	5	6	Total asci.
1	40	36	24	28	35	24	187
2	17	11	8	16	8	13	73
3	20	19	18	18	19	23	117
4	31	19	27	21	22	15	135
Total	108	85	77	83	84	75	512

The frequencies in classes 1 and 2 do not differ significantly from equality (χ^2 is 2·74 for 1 degree of freedom). The percentage of second division segregation is 62·3, which does not differ significantly from 66·7 per cent., the value obtained when the locus is so far from the centromere that it segregates independently of it. There appears, therefore, to be no polarization of segregation such as was present in Zickler's data for *Bombardia lunata* (Catcheside, 1944).

The joint segregation of spore colour and spore size was scored in 70 asci from the cross *al–1* $l^+ \times +$ *l*. There were 67 non-recombinant and 73 recombinant half asci. The loci for *albino–1* and spore size are therefore not linked.

The rate of mutation. It is not possible to determine the mutation rate when there is no method available for detecting an individual mutation, but if each stroma equals 1 mutation, the rate must approximate to $50/n$, where n is the number of nuclei present in a culture, probably greater than 10^6. Apart from being impracticable, isolation of hyphal tips would not give a true estimate of the mutation rate. To exclude a mutation rate as high as 0·15 per cent. at the $P = 0{\cdot}05$ level, it would be necessary to isolate 1,000 hyphal tips.

DISCUSSION

There are two ways in which the mating behaviour of *Chromocrea* might be interpreted. It may be a homothallic fungus which is heterozygous for a

sterility factor or it is heterothallic and the self-fertility of half the spores in the ascus is caused by a mutation in mating type which occurs only in cultures from these particular spores.

If the fungus is homothallic it is difficult to explain the continual recurrence of the sterility factor in the progeny of spores from which it is absent. To postulate a mutation here does not overcome the difficulty. In a heterothallic fungus every ascus must be heterozygous for mating type. In a homothallic fungus, however, any two nuclei may be associated in the formation of the perithecium, and if the fungus is heterocaryotic it is purely a matter of chance whether like or unlike nuclei are involved. Obviously there is no mechanism in such a fungus for assuring that each perithecium is hybrid for any particular factor, especially for one that determines sterility of cultures grown from half of the ascospores.

The segregation of spore size which takes place in every ascus developed in single l^+ cultures in *Chromocrea* shows that the fungus is heterothallic and spore size is therefore a morphological expression of mating type. The presence of both mating types in such cultures can result only from mutation.

When both types of spore give rise to colonies which are self-sterile as in some of the morphological mutants of *Chromocrea*, the new mutation may either be inhibiting the mutation in mating type or preventing the formation of perithecia after it has taken place. If the former situation existed and if there was no block in the formation of perithecia it would be expected that a cross between a large and a small spore colony of the mutant would be fertile. The mutant *fluffy* may come into this category although perithecial formation is reduced even when both mating types are present. The mutants *irregular* and *dense* belong to the second category and are only fertile when crossed with wild type. The mutant *restricted* obviously also belongs to this group, but whereas in the case of *irregular* and *dense* the heterocaryotic condition for mutant and wild type nuclei must be preserved throughout sexual reproduction, in the case of *restricted* the substance or substances produced by the wild type colony and required by *restricted* for normal perithecial development are apparently diffusible, either through the medium or along the hyphae, as selfed perithecia often develop at the edge of the mutant colony adjoining the wild type.

Whitehouse (1949) lists 37 species of Ascomycetes belonging to 8 different orders in which physiological heterothallism has been recorded. In all those species on which adequate tests have been carried out, the heterothallism is of the two allelomorph type. In general the mating type alleles are extremely stable. In *Neurospora* where many thousands of asci have been analysed no mutation of mating type has ever been recorded although loss of fertility may occur when stocks are kept in culture.

There are, however, a few exceptions in which mating type appears to be unstable. As a result of this instability sexual reproduction can occur in single ascospore cultures. This may be due either to a mutation at the mating type locus to the other allele and subsequent fusion of unlike nuclei, in which case

the resulting asci should show segregation for both mating types, or to a so-called 'illegitimate' mating in which nuclei of the same mating type fuse and give rise to asci which contain ascospores all of the same mating type. The latter type may result from inactivation of the self sterility allele either through mutation at that locus or through a suppressor.

Glomerella cingulata shows some similarities to *Chromocrea* but the situation appears to be more complex. In a series of papers by Lucas et al. (1944), Edgerton et al. (1945), Chilton et al. (1945), Chilton et al. (1949), and Markert (1949) the mating behaviour of this fungus has been described. On the basis of their behaviour in crosses strains of *Glomerella* have been divided into two groups, (+) and (−), which when grown together form a ridge of perithecia on the line of contact. Single spore cultures of (+) strains are self-fertile and the asci in individual perithecia are either hybrid for (+) and (−), all (+) or all (−). This behaviour suggests a mutation in mating type, but the fact that these three different types of perithecia are formed would indicate that *Glomerella* resembles a homothallic fungus which has become heterocaryotic for (+) and (−) characters by mutation. Evidence given by Markert (1949) on mating behaviour in *Glomerella* shows that diffusible chemical substances determine the degree of fertility of any cross. These substances are no doubt controlled genetically, but there is no evidence that the fungus is heterothallic in the sense that mating is controlled by a single pair of alleles.

The only well-established case in Ascomycetes of a mutation from one mating type to the other has been described for species of *Saccharomyces* (Ahmad, 1948). Lindegren and Lindegren (1943, 1944) have shown that mating in strains of *S. cerevisiae* is normally controlled by a single pair of alleles a/α which segregate in the ascus in the usual way. They also found that some colonies would not mate with either of the tester strains and considered that the mating type alleles were unstable or else mutation of modifying genes was inhibiting mating. In their later work on biochemical mutants of yeasts they have selected for stability of mating type, and in most of their pedigrees the *a* and α alleles segregate normally.

Ahmad used Danish Baking yeast, a strain of *S. cerevisiae*, and found that heterothallism was controlled by a pair of alleles. Nevertheless a high proportion of his single ascospore cultures gave no reaction with either tester strain but sporulated quite readily, thus behaving as though they were homothallic. When the asci were dissected it was found that the mating type factors had segregated in the ascus and it was postulated that the fertility of such single ascospore cultures was due to a mutation in mating type. By taking a series of single cell isolates from an *a* strain and another series from an α strain and testing their reaction, Ahmad found that mutation in mating type could take place in either direction, i.e. from *a* to α, or from α to *a*. The same sort of mating type instability was shown by *S. carlsbergensis* and a strain designated *S. sp* Form 237.

The situation is slightly different in *Chromocrea*, for here the mutation appears to take place in one direction only. This might be taken to indicate

that the mating type allele of the small spores is more stable than the allele of the opposite mating type, as the mutation is apparently irreversible under the conditions existing in the mycelium. There is no evidence that the mutation is controlled by any particular gene nor by the genotype as a whole, since this must be the same for both types of spore when they have come from a single l^+ spore culture. This may not hold for the morphological mutants although the evidence here is in favour of the particular mutation inhibiting the development of perithecia rather than the actual mutation of mating type. *Fluffy* may be an exception.

A number of cases of dimorphism have been recorded in the Ascomycetes. In *Phaeobulgaria inquinans* four dark and four smaller colourless spores are formed in each ascus. Whitehouse (1947) has shown that the spore character is governed by a pair of alleles, but since he was unable to obtain apothecia in crosses it is impossible to tell whether or not the dimorphism is associated with mating type.

Segregation of factors affecting spore colour has been recorded by Dowding (1931) for *Ascobolus stercorarius*, by Rizet (1934) for *Ascobolus immersus*, and by Zickler (1934) for *Bombardia lunata*, while Wülker (1935) described the segregation of a factor affecting the rate of ripening of spores in crosses between *Neurospora crassa* and *N. sitophila*. Differences in spore size in the secondarily homothallic species, *Neurospora tetrasperma* (Shear and Dodge, 1927), *Gelasinospora tetrasperma* (Dowding, 1933), and *Pleurage anserina* (Dowding, 1931) are directly related to the number of nuclei included in the spore when it is first delimited. Dwarf spores contain one nucleus and are unisexual, while normal spores with two nuclei and giant spores with more than two are secondarily homothallic. In none of these cases, however, is the spore dimorphism associated with mating type as it is in *Chromocrea*.

Whitehouse (1949) has discussed the significance of heterothallism and homothallism in the fungi and has suggested the possible evolutionary steps by which these conditions may have arisen. He has shown that there is a graded series amongst the physiologically heterothallic Ascomycetes from forms in which heterocaryosis is restricted to nuclei of the same mating type, such as *Neurospora crassa*[1] and *N. sitophila*, to those in which heterocaryosis may involve nuclei of both mating types. This latter group can be subdivided into a group in which sex organs are differentiated but are not essential for sexual reproduction as in the secondarily homothallic species, and a second group where sex organs are not differentiated.

Chromocrea appears to be intermediate between these last two groups. It is quite clear that heterothallism of the two allelomorph type exists and that heterocaryosis is unrestricted. Male sex organs at least are not differentiated and female sex organs have not been seen. If they are present they must be functionless as such, and nuclei of opposite mating type are probably brought together by hyphal fusion followed by migration of nuclei at some stage

[1] S. R. Gross (1950) has recently shown that heterocaryosis will occur between opposite mating types in *Neurospora crassa*.

before the formation of the ascogenous hyphae. The large ascospores give rise to a culture which is essentially secondarily homothallic as the result of a mutation in mating type and not by the inclusion in a single ascospore of nuclei of opposite mating type as in *N. tetrasperma*. The small spore cultures are strictly heterothallic. As there are no asexual spores the secondarily homothallic character of the l^+ spores ensures that reproduction can always take place, while the advantages of outbreeding conferred by the facultative heterothallism are still retained. The unrestricted heterocaryosis increases the plasticity of the organism.

Summary

The asci of *Chromocrea spinulosa* (Fckl.) Petch, *in litt.*, contain four large and four small ascospores each two-celled. These are arranged in the six ways expected if spore size is controlled by a pair of allelomorphs. Each original ascospore divides into two separate spores before discharge.

The larger ascospores give rise to colonies which are self fertile, producing asci which again show segregation for spore size. The smaller ascospores give rise to colonies which are sterile.

The production of perithecia is stimulated at the junction of a colony grown from a large ascospore and a colony grown from a small ascospore; but since the only marker is spore size it is impossible under these conditions to identify hybrid asci.

A number of morphological mutants were isolated after treatment of wild type with ultra-violet light and X-rays. These differ from wild type in the nature of the mycelial growth or in the colour of the ascospores.

By crossing these mutants with wild type it is possible to show that the difference in spore size is a pleiotropic expression of mating type, and that the fungus is heterothallic. The fertility of the colonies from large spores, then, can only be the result of mutation in mating type which is expressed in the dimorphism of the ascospores.

Heterocaryons are formed very easily and irrespectively of the original mating type of the two components.

Using mutants affecting both mycelial characters and ascospore colour, it is possible to show that nuclei are able to migrate freely through the mycelium of another strain, but that after a mutation in mating type, the mutated nuclei do not migrate before fusing or at least becoming associated with nuclei of the opposite mating type.

An ordered tetrad is formed in the ascus of *Chromocrea*. Stained preparations of asci suggest that there are eight bivalents at metaphase I. The chromosomes, however, are so small that this cannot be stated with certainty. In general the constitution of the asci in each perithecium is determined by a single primordial pair of nuclei, but occasionally mixed perithecia are found when one or other of the mycelia involved is heterocaryotic.

Since the segregation of the characters for spore size and colour can be observed directly in the ascus, it was found possible to determine the

frequencies of the six arrangements for each character. There is no evidence of biased segregation.

I should like to express my gratitude to Professor F. T. Brooks and Professor D. G. Catcheside for their help and guidance, also to the British Council and the University of Melbourne for financial assistance. The work was done while the writer was on leave from the University of Melbourne. I am indebted to Mr. H. Elborn for the photographs.

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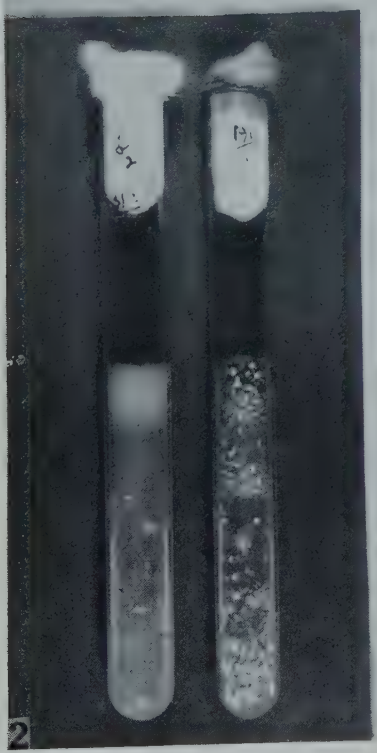


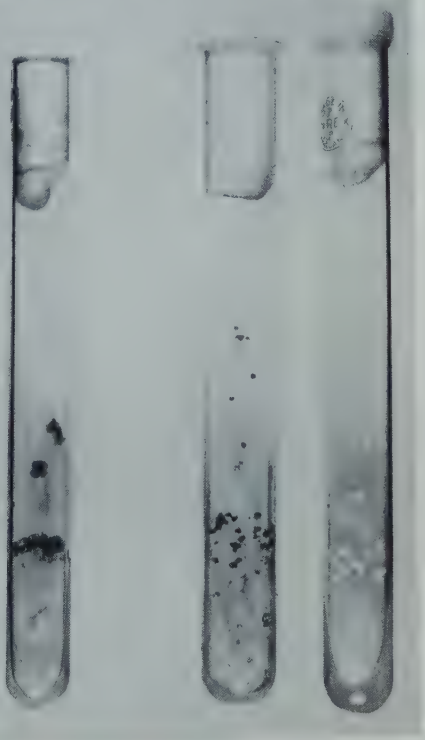
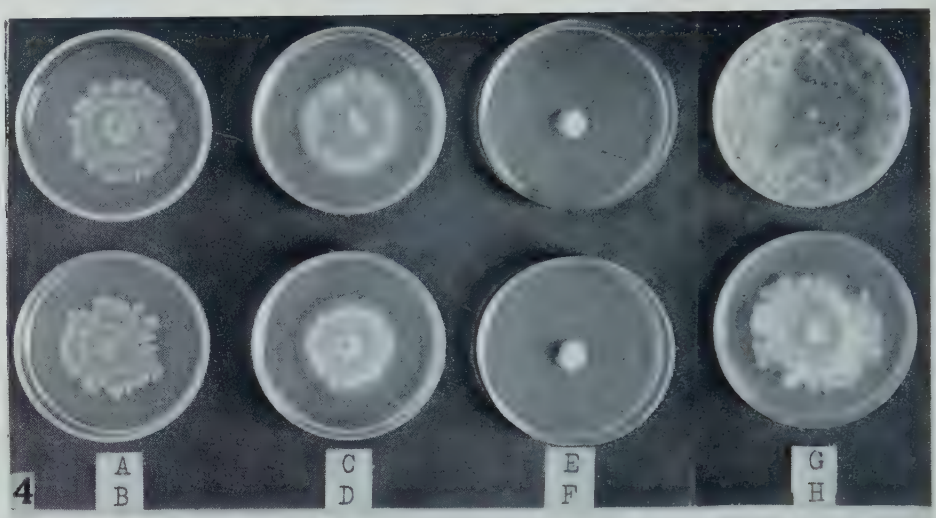


FIG. 1. Wild type asci showing segregation of the factor controlling spore size. ×500

FIG. 2. Left. Slope culture from a single l^+ ascospore. Right. Slope culture from mixed l^+ and l ascospores

FIG. 3. From right. Slope cultures of *albino–1* l^+, wild type l^+, and a cross between wild type l^+ and *albino–1* l, with wild type above and *albino* below



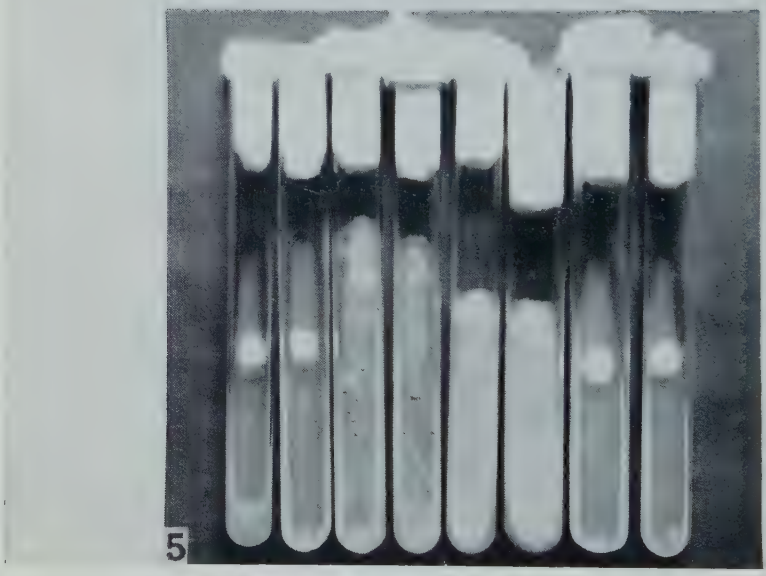


FIG. 4. Morphological mutants

A. *irregular l+*
B. *irregular l*
C. *restricted l+*
D. *restricted l*
E. *dense l+*
F. *dense l*
G. *brown exudate l+*
H. *fluffy l+*

FIG. 5. Progeny from a single hybrid ascus developed from a cross between *dense l+* and wild type *l*

From left: *den l+*, *+l+*, *+l*, *den l*

Some Features of Root Growth in Nodulated Plants of *Myrica gale* L.

BY

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With Plates XXIII and XXIV, and two Text-figures

Abstract

The roots which are produced in large numbers from the root nodule clusters in *Myrica gale* have been observed by the author to be characterized by vertically upward growth under a wide range of cultural conditions, evidence having been obtained that the same is true in the field. Experimental investigation indicates that the orientation is the result of negative geotropism. Structural and experimental evidence permits the view that these upward-growing roots facilitate the ventilation of the nodule tissues. This is likely to be of special significance under the bog conditions in which the species commonly grows.

Introduction

In an investigation already reported (Bond, 1951*b*), evidence strongly indicative of the occurrence of fixation of atmospheric nitrogen in association with nodulated plants of *Myrica gale* was obtained. In that investigation nodulated plants were grown in water culture, and in such plants the orientation assumed by the roots, especially those which, as described by previous authors, grow out from the tips of the nodules, showed unusual features. These growth-orientations in what, in this paper, will be termed the 'nodule-roots' do not appear to have been noted by any previous investigator and were considered to merit further study, particularly since it seemed possible that they were of functional significance to the nodule, which is presumably the seat of the nitrogen fixation.

[Annals of Botany, N.S. Vol. XVI, No. 64, October 1952.]

The Development and Orientation of the Nodule-roots in Water Culture

As indicated in the previous paper (Bond, 1951*b*), the procedure adopted for obtaining nodulated plants in water culture was to transplant seedlings at the 2- to 4-leaf stage from peat into water culture (free of combined nitrogen) and shortly afterwards to apply to the roots a suspension of crushed nodules from other plants. Visible nodules appear 2 to 3 weeks after the inoculation, and nodule-roots grow from these nodules after a further 2 weeks. New lobes soon develop on the originally simple nodules, leading to

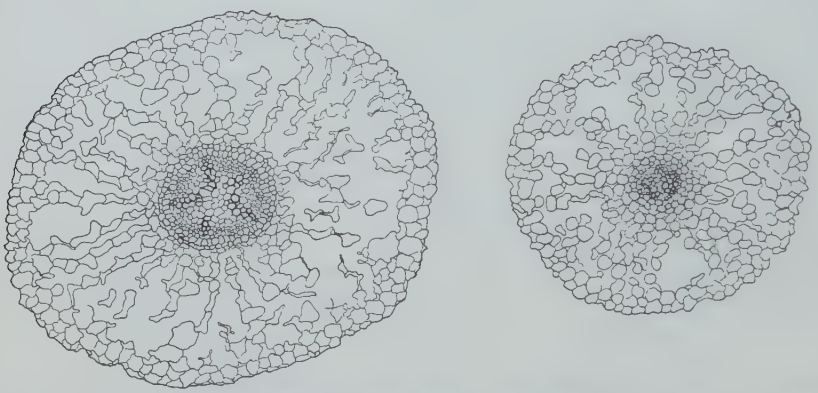

Fig. 1. Structure of normal root (left) and nodule-root (right) as shown in transverse section 3 cm. from the tip, in water culture material. (× 85.)

the formation of nodule clusters, from the tip of each lobe of which a nodule-root grows out. These developments will be described in more detail in a later paper from this laboratory.

It was observed that in water culture the nodule-roots, as soon as they emerge from the nodule, turn upwards and continue to grow in that direction. The root system of a young plant thus presents a rather curious appearance, as shown in Pl. XXIII, Figs. 1 and 2. It will be noted that at this stage the upward-growing nodule-roots account for a large part of the total root system. They attain, during the first year of plant development in water culture, a length ranging up to 4 cm., and the roots growing from nodules situated high up on the root system usually emerge from the culture solution, this being followed eventually by a withering of the tip and cessation of growth.

In older plants growing in water culture the nodule-roots continue to be very conspicuous. Pl. XXIII, Fig. 3, shows the root system of a typical second-year plant. If, as often happens, further nodule clusters develop lower down on the root system, these too soon come to be attended by tufts of upward-growing roots (Pl. XXIII, Fig. 4). In these second-year plants the nodule-roots often attain a length of 8 cm.; their origin and relation to the nodules are clearly shown in Pl. XXIII, Fig. 5.

The nodule-roots typically remain unbranched, but the occasional production of laterals has been noted. Root-hairs occur sporadically on the nodule-roots, as they do also on the normal roots in water culture. Text-fig. 1 indicates the structure of both types of root as shown in transverse section. The stele is relatively smaller in the nodule-root, but otherwise the latter is similar to the normal root, both showing extensive air-spaces in the cortex. It may be noted here that nodule-roots from field material show a similar structure to that figured for water culture material, except that the extent of the cortical air-spaces is more variable, a small proportion of the numerous specimens examined showing a more compact cortex with smaller air-spaces. Bottomley (1912) stated that the nodule-roots have no definite root-cap, but this cannot be confirmed since all such roots examined by the present author have shown clear root-caps. No invasion of the tissues of the nodule-roots by the nodule endophyte has been observed.

The Orientating Factor

Attention has been given to the identity of the orientating factor productive of the upward growth of the nodule-roots. As will appear below, the investigation of this problem has led to observations on root behaviour in other media, but it will be convenient to start by considering water culture conditions, in respect of which the following possibilities have been entertained:

(*a*) the upward growth might be due to negative geotropism;
(*b*) the upthrust experienced by roots of low specific gravity growing in an aqueous medium might direct growth upwards if positive geotropic tendencies were only feebly developed;[1]
(*c*) the direction of growth might be an aerotropic response, governed by oxygen gradients;
(*d*) positive phototropism might be responsible;
(*e*) the phenomenon might be due to an inherent, autotropic tendency for the roots to orientate themselves parallel to the main root but pointing in the opposite direction.

In connexion with the possibility of *aerotropic response*, it should be recalled that the water cultures in which these features of root growth have been mostly studied have been of unaerated type, with an air space present between the teak top and the surface of the culture solution. Under such conditions a concentration gradient of dissolved oxygen may be expected to establish itself in the culture solution as root growth and respiration become appreciable. Observations have shown, however, that the upward growth of nodule-roots becomes manifest before any oxygen gradient can be detected.

[1] Determinations of the specific gravity of samples of nodule-roots gave values of the order of 0·7. A few tests on normal roots, again from water culture plants, showed higher values (region of 0·9), but no further comparisons were made after it became clear that, for reasons to be indicated below, upthrust was not responsible for the upward growth.

Thus estimations of dissolved oxygen by micro-Winkler technique were carried out at successively lower levels in jars containing small plants, the nodule-roots of which, though still short, were already showing marked upward growth. No measurable gradient of oxygen could be detected in these jars, all samples showing a dissolved oxygen content close to the equilibrium figure for water in contact with air. Other experiments have shown that the upward growth is still exhibited when definite measures are taken to eliminate oxygen gradients, for example, by supplying forced aeration or by using a constantly flowing solution technique. Finally, the evidence to be presented below on root behaviour in non-fluid media (particularly in moist air) confirms that the upward growth is not a response to oxygen gradient.

Though the exhibition of *positive phototropism* by root structures would be very unusual, it seemed necessary to consider this possibility since the cultural arrangement normally employed allowed a little light to fall on the root system from above. The critical experiment of growing the root system in absolute darkness has not been performed, but in a few special cultural arrangements (e.g. that used in the klinostat experiments—see below) any slight amount of light falling on the roots came from one side. The root orientation showed no relation to this, and for this reason, together with the fact that the upward growth is shown in opaque rooting media such as sand (see below), it is concluded that phototropism is not involved.

A response to *upthrust* would be limited to solution culture, and this consideration naturally led to investigation of root behaviour in other rooting media. In one trial 50 seedlings were transplanted into pots containing moss litter (chiefly *Sphagnum* spp.) collected from underneath bog myrtle plants in the field, and the seedlings then inoculated. The pots were watered from below, Crone's solution, free of combined nitrogen, being supplied occasionally. The plants grew very vigorously and showed abundant nodule and nodule-root formation. In all plants the nodule-roots grew upwards, those from the higher nodules reaching the surface of the moss (Pl. XXIV, Fig. 7).

In other tests it was found that upward growth of the nodule-roots was also shown in sand culture and in moist air. The latter statement is based on an experiment with second-year plants from which, at the commencement of the test, all existing nodule-roots were excised, the level of the solution in each jar being at the same time lowered to 10 cm. below the teak top, so that the main nodule mass was well clear of the solution and exposed to moist air only. Within a few days' time new nodule-roots appeared and grew upwards (Pl. XXIII, Fig. 6).

It is thus established that the upward growth is not exhibited in fluid rooting media only. It is therefore not due to upthrust.

It is convenient at this point to refer to the orientation of the nodule-roots under field conditions. Considerable difficulty is experienced in getting evidence on this point, because of the small, fragile nature of the roots. The impression gained from examination of material in the field in autumn and winter is that the nodule-roots project from the nodule clusters in all direc-

tions, but a different picture is seen in the summer months when crops of newly formed nodule-roots are present. It is then clear that in any particular nodule cluster the nodule-roots all tend to grow in the same direction (Pl. XXIV, Fig. 8). In several instances it was possible, by referring to the suckers to which nodule clusters were attached in dug-up material, to establish the original orientation of the clusters in the rooting medium, and in each case it was clear that the nodule-roots had been growing in an upward direction (Pl. XXIV, Fig. 9). Taking these observations along with the

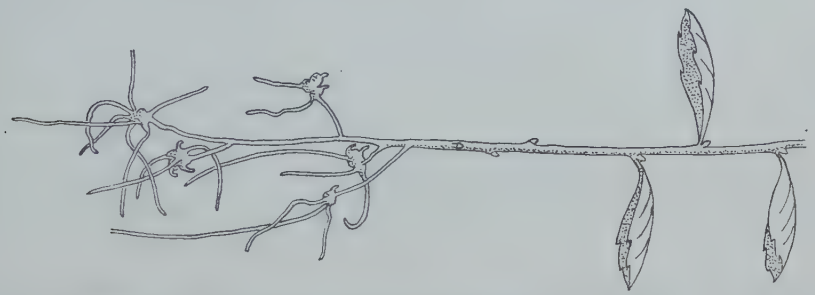

FIG. 2. Appearance after 2 months' growth on klinostat of plant placed horizontally and rotated about its long axis. Part of the shoot and some of the roots unconnected with nodules are omitted. (Nat. size.)

evidence obtained under experimental conditions that the upward growth of the nodule-roots is a fixed character, it becomes extremely probable that more widespread examination will confirm that these root features are regularly shown under field conditions. The fact, already mentioned, that the characteristic orientation is less clearly shown in older material may well be due to shrinkage and settling movements of the peaty rooting medium consequent on changes in water content.

Of the possible orientating factors listed above, only *negative geotropism* and *autotropism* now remain for consideration, and to investigate these further, klinostat experiments were carried out. Three plants were grown in water culture on a klinostat for a period of 2 months, being placed in a horizontal position and revolved about their long axes once every 25 minutes. A similar number of control plants in a fixed horizontal position was also set up, and from all plants any existing nodule-roots were removed at the commencement. In the control plants the new nodule-roots grew upwards as shown in Pl. XXIV, Fig. 10.[1] The appearance, at the end of the growth period, of the plants rotated on the klinostat is shown in Text-fig. 2. The nodule-roots had grown in various directions, indicating that the rotation had nullified the effect of the orientating factor. The results of these experiments show that the upward growth of the nodule-roots under normal circumstances is not due to an inherent, autotropic influence. Taken in conjunction

[1] Actually this photograph is of a plant from a different experiment, not reported in the present paper, but the general circumstances of growth were identical with those of the klinostat control plants.

with the previous evidence they indicate that the upward growth is due to negative geotropism.

The Role of the Nodules in Inducing Upward Root Growth

If it could be shown that the upward growth was strictly confined to the nodule-roots and was not exhibited by any of the normal roots unconnected with nodules, then it would seem probable that the presence of the nodules was in some way responsible for the phenomenon. In young nodulated plants, such as those shown in Pl. XXIII, Figs. 1 and 2, the feature is confined to the nodule-roots, but after the root system has become more extensive roots unconnected with nodules sometimes show upward growth in water culture. An instance of this is provided in Pl. XXIV, Fig. 11, where marked upward growth is shown by a number of second-order lateral roots in the lower part of the photograph. Upward-growing roots have also been observed in plants free of nodules and supplied with nitrate-nitrogen, a striking instance of this being provided by a second-year plant of this type which, in respect of root orientation, showed a close resemblance to a nodulated plant (Pl. XXIV, Fig. 12).

Summarizing the experience gained over several years' work with *Myrica gale* in water culture, the position is that the nodule-roots always show upright growth, while other roots of higher orders frequently show the same feature, presumably again as the result of an assumption of negative geotropism. It is concluded that the great prominence of upward root growth in the nodular regions is partly due to the precocious and rapid production of roots of successively higher orders in these regions, such roots tending, independently of the presence of the nodules, to negative geotropism. So unfailing is the response in the nodule-roots, however, that it seems necessary to conclude that in some way the presence of the nodule reinforces any innate tendency of the roots to grow upwards.

Any attempt to pursue further the causal mechanism of these root phenomena in *Myrica gale* would lead to a consideration of the tropic status of lateral roots in general, which is beyond the scope of the present paper. It may, however, be noted that assuming an auxin mechanism for geotropism it is conceivable that as a result of metabolic activities within the nodule there might be modifications in the amount or nature of the growth-substances present in the nodule-roots.

The Functional Significance of the Nodule-roots

It has finally to be considered whether the nodule-roots have any special functional significance, and whether the upward growth contributes to the efficient discharging of any such function.

Since they constitute such a large part of the root system, especially in young plants, it must be concluded that in the first place the nodule-roots perform normal root functions for the benefit of the plant as a whole.

In addition they may well have a particular significance for the nodules.

In his account of the structure of the nodules of *Myrica gale*, Chevalier (1900–2) stated that at an early stage in its development the nodule becomes enclosed by a superficial cork layer. His description indicates that the cork layer is forming prior to the emergence of the nodule-root, a finding confirmed in the present author's laboratory by Mr. W. Fletcher. This cork covering will tend to isolate the nodule from direct communication with the environment,[1] but when the nodule-root grows out it will provide a new channel of communication, shorter than that provided by the connexion with the main root system. These new channels will no doubt serve for gaseous exchange, and in fact the nodule-roots may perhaps be regarded as constituting pneumatophores, each serving one nodule lobe. It should be noted, however, that apart from the abundant air-spaces the nodule-roots show no special structural features, such as lenticel formation, for facilitating gas exchange.

Although it is reasonable to assume that the nodule is the centre of considerable metabolic activity, it does not follow that there will be a high oxygen requirement. The importance of oxygen to the nodulated root system has been investigated by a technique similar to that previously employed for legumes (Bond, 1950*a*), in which plants are grown in water culture with the culture solution maintained in equilibrium with different oxygen/nitrogen mixtures. Already-nodulated plants, 2 months old, were sealed into culture jars of the type used previously, and growth observed for a period of 2 months during which the appropriate gas-mixtures were bubbled through the culture solution constantly. Data relating to the plants are presented in Table I.

TABLE I

Effect of Oxygen Tension on Growth of Nodulated Plants of Myrica gale*

% oxygen in gas passed.	Mean increase in height of shoot during period (mm.).	Relative mean area of single leaf at harvest.	% nitrogen in leaf dry matter at harvest.
21	12 (6–18)	100	3·60
12	10 (5–17)	84	3·41
5	10 (3–19)	80	3·02
1	5 (1–14)	68	2·38

* Nine plants were grown at each oxygen level, in Crone's solution free of combined nitrogen, pH 5·4. Growth period August 18 to October 18. The figures in parentheses in the second column indicate the extreme values. The data in the third column are based on fresh weight of the leaves, this being taken as an index of area.

In addition to the numerical data shown in the table it was observed that the stems and nodules of the 1 per cent. oxygen series soon developed intense red pigmentation, while the leaves of the same series, and to a lesser extent those of the 5 per cent. series, soon became chlorotic. In the same two series root development was relatively inferior.

[1] A somewhat comparable state of affairs exists in the legume nodule owing to the presence of suberized endodermal sheaths in the outer regions of the nodule (Frazer, 1942).

The above evidence indicates that the curtailment of growth contingent on the reduction in oxygen supply, particularly marked in the 1 per cent. series, was due primarily to restriction in fixation of nitrogen, resulting in symptoms of nitrogen deficiency. It may be concluded (*a*) that the fixation is a distinctly aerobic process, and (*b*) that the diffusion of oxygen from the shoot to the root system, if it occurs at all, is inadequate to prevent a severe reduction of growth when the root system is exposed to low oxygen tension, suggesting that the root system is normally self-supporting as regards oxygen intake.

Under the bog conditions in which *Myrica gale* typically grows, the oxygen content of the rooting medium is likely to fall frequently to levels that are critical for root activities in general and for the fixation process in particular. Although the nodule-roots in field material have not been observed to exceed 4 cm. in length, many of them being considerably shorter than this, it is obvious that their upward growth will provide access to regions of higher oxygen content than would be available to normally orientated roots, and it is possible that in this way the efficient functioning of the nodules is promoted to a significant extent.

Summary

It is shown that the roots ('nodule-roots') which spring from the lobes of the root nodules in *Myrica gale* grow upwards in water and sand culture, also in moss litter and in moist air. These observations lead to the expectation that the feature will also be shown under field conditions, as has been confirmed in a number of instances.

Evidence is presented indicating that the upward growth represents a negative geotropic response.

Though under cultural conditions the upward growth is sometimes shown by normal roots unconnected with nodules, the great regularity with which the feature is associated with the nodule-roots suggests that the presence of the nodules is in some way partly responsible for the assumption of negative geotropism by the roots growing from them.

Since the nodule becomes enclosed by a layer of cork at an early stage, it is likely that the nodule-roots provide the main channel of communication between the interior of the nodule and the environment, especially for gaseous diffusion.

It is shown that under experimental conditions the fixation of nitrogen is curtailed by a reduced external oxygen supply to nodulated roots, and it is probable that there will be a tendency for limitation of fixation by this factor under the bog conditions in which *Myrica gale* commonly grows. The upward growth of the nodule-roots is likely to improve the supply of oxygen to the nodule tissues.

The author wishes to acknowledge the assistance in experimental work given by Miss A. H. McCallum and Mr. J. MacArthur, and that in photography by Mr. W. Anderson.

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EXPLANATION OF PLATES

Illustrating G. Bond's article on 'Some Features of Root Growth in Nodulated Plants of *Myrica gale* L.'.

PLATE XXIII

Fig. 1. Nodulated plant grown for 3 months in water culture. (× ½.)

Fig. 2. Nodulated plant grown for 3½ months in water culture, root system photographed under water. (× ⅓.)

Fig. 3. Nodulated plant early in second year of growth in water culture, root system photographed under water. (× ¼.)

Fig. 4. As in Fig. 3, but a different plant, showing additional loci of nodule development. (× ¼.)

Fig. 5. Detached portion of root system of a second-year plant, in original orientation, showing nodules and nodule-roots. (Nat. size.)

Fig. 6. Root system of one of the plants of the moist-air experiment. The upward-growing roots at the centre of the photograph assumed this position although growing in moist air only. (× ¼.)

PLATE XXIV

Fig. 7. The lower part of the shoot and part of the root system of a nodulated plant grown for 3 months in moss litter. (Nat. size.)

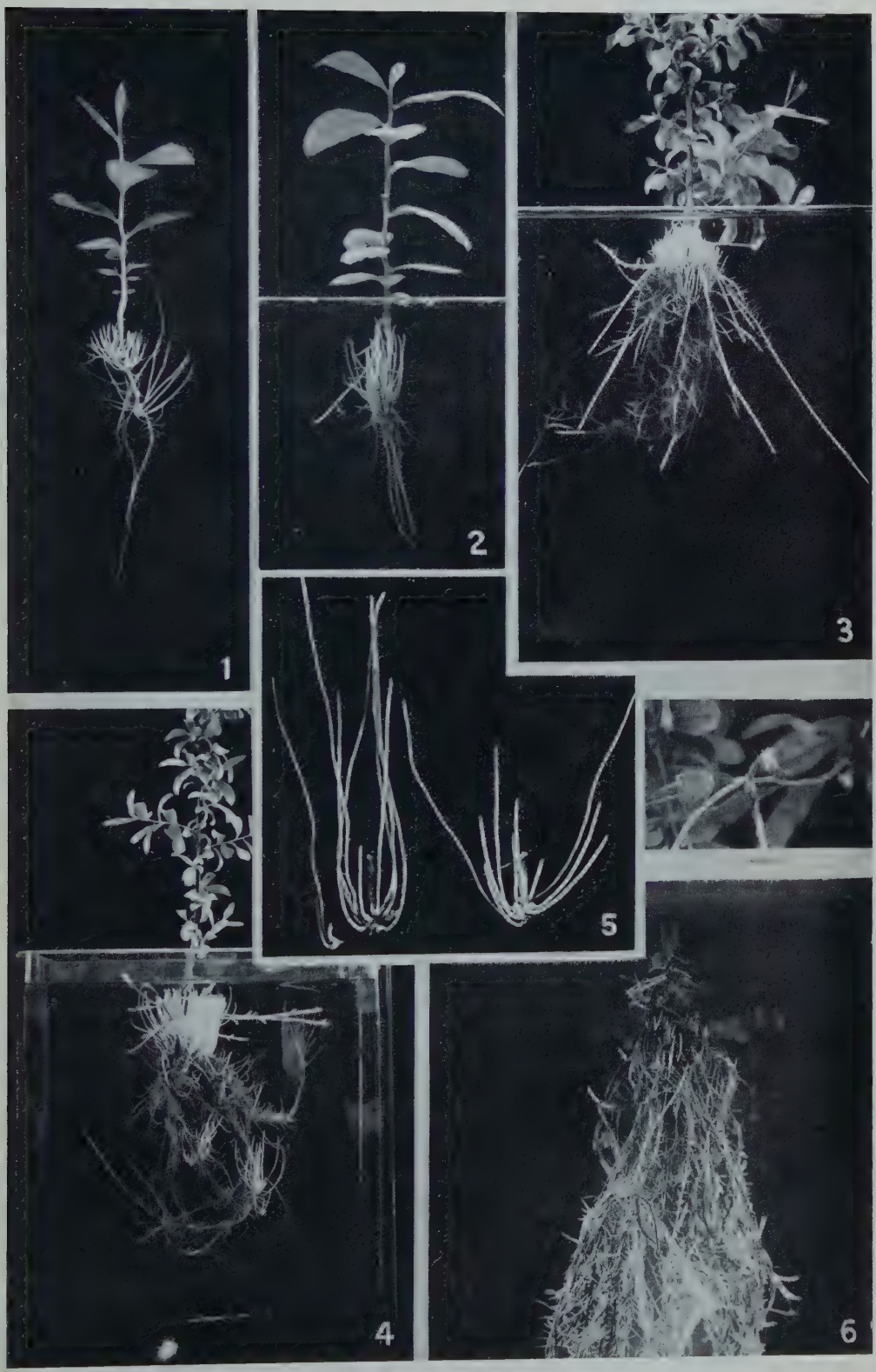
Fig. 8. Nodules and nodule-roots from field material, collected in June, original orientation unknown. (Nat. size.)

Fig. 9. Portion of sucker with attached nodules and nodule-roots, from field material collected in June, in original orientation. (× 2½.)

Fig. 10. Nodulated plant grown in water culture and fixed initially in a horizontal position. Photographed 3 months after commencement of the experiment. (× ½.)

Fig. 11. Nodulated plant grown for 4 months in water culture, root system photographed under water. The nodule-roots forming the tuft near the base of the stem had become bent over through contact with a rubber stopper in which the plant was fixed. The plant received a small amount of combined nitrogen at an early stage. (× ¼.)

Fig. 12. Root system of non-nodulated plant supplied with nitrate-nitrogen, in second year of development in water culture. (× ⅛.)



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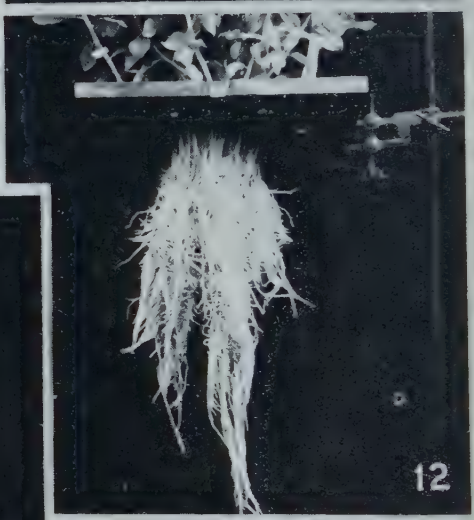
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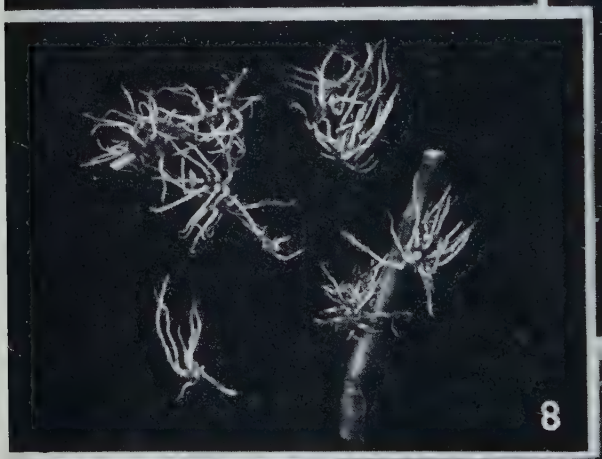
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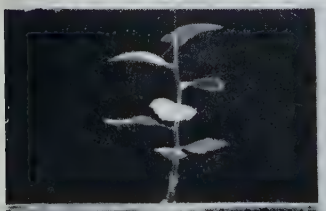

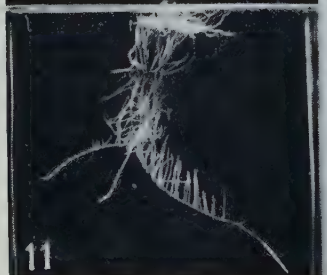
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Investigations on Growth and Metabolism of Plant Cells

III. Evidence for Growth Inhibitors in Certain Mature Tissues

BY

F. C. STEWARD[1]

AND

S. M. CAPLIN[2]

With five Figures in the Text

ABSTRACT

Using the carrot explant technique, with added coconut-milk factor, evidence has been obtained of inhibitors of growth in potato tubers, onion bulbs, and maple buds. In discussion it is suggested that the regulation of growth in such tissues as mature parenchyma may thus be due to absence of necessary growth-factors or the presence of growth inhibitors.

THE growth-promoting qualities of coconut milk which enable rapid proliferative growth of the secondary phloem of carrot root to be obtained under tissue-culture conditions are due to a heat-stable and water-soluble substance or substances designated, for convenience, the coconut-milk factor (C.M.F.). A great deal of evidence has indicated that, although this activity may be obtained elsewhere in the plant kingdom, e.g. from the immature fruits of corn (*Zea mays*) and also from the developing gametophyte of the maidenhair tree (*Gingko biloba*), it is not commonly to be found in such obvious sources of potent vitamin and growth-promoting activity as yeast, liver, tomato juice, and casein hydrolysate, though the latter contains compounds which increase the growth of carrot tissue, the effect when found being *additive* to that of the coconut milk. Nor is C.M.F. identical with a large number of distinctive substances which have been tested because of their well-known biological activity in other directions. In advance of exact knowledge of the biochemistry of the coconut-milk factor or factors it is, however, profitable to discuss their regulatory role in the systems of which they form a part. This discussion falls logically into the three parts, to be indicated below.

Growth inhibitors

Carrot secondary phloem is an example of a tissue in which the already differentiated cells will return to the dividing proliferating state if they receive,

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[Annals of Botany, N.S. Vol. XVI, No. 64, October, 1952.]

in addition to the substances (White, 1943) often furnished in nutrient tissue-culture media, materials to be found in coconut milk. Other mature cells, e.g. cells of the artichoke tuber, grow as well as carrot tissue in this combination of nutrients and growth-factors. Fig. 1 shows the relative growth obtained with explants from various storage organs when these were allowed to grow in the basal nutrient medium supplemented with coconut milk for a period of 21 days. The growth obtained under the conditions of the rotating liquid cultures (Steward, Caplin, and Millar) was, for all of the storage organs, consistently superior to the other conditions tried (on agar in stationary or rotating

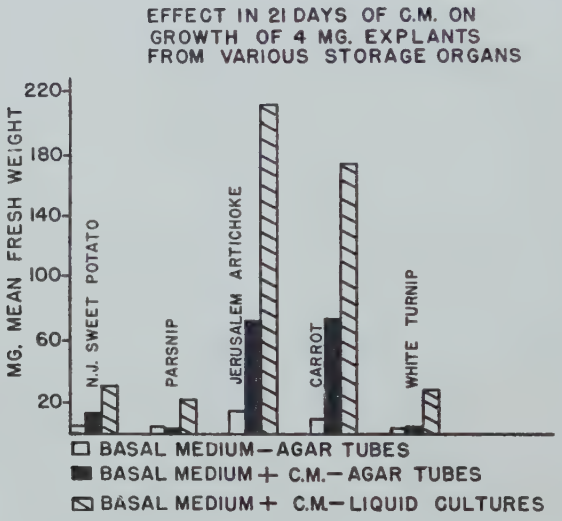

Fig. 1

tubes). Explants from the Jerusalem artichoke tuber (*Helianthus tuberosus* L.) grew with comparable vigour to the carrot root, and the growth of these two far exceeded that of explants from the other storage organs which responded to various degrees, viz. sweet potato (*Ipomea Batatas*, Lam.), parsnip (*Pastinaca sativa* L.), white turnip (variously called *Brassica campestris* var. *rapa* or *B. rapa*). Still other cells, e.g. of the potato tuber, are completely unable to grow in these media because their activity is still further regulated in ways which prevent the tissue from responding by proliferating to the combined effect of the nutrient solution and its coconut-milk supplement.

This situation, in which some cells fail to grow under what would seem to be ideal nutrient conditions, leads to the concept that such cells may fail to grow because of some regulatory mechanism or inhibitor for the coconut-milk factor and that this is a characteristic of their mature state. Hence the direct question to be answered is whether the failure of many living mature parenchyma to proliferate and grow rapidly, even under what seem to be ideal nutrient conditions, may not be due to 'anti-growth factors' which inhibit,

or prevent the action of, the factors for proliferative growth which are to be found in coconut milk.

Just as the system composed of standard explants of carrot tissue may be used to assay the growth-promoting factor, so it may also be used to detect inhibiting substances or mechanisms if these are added to the nutrient containing carrot explants and the optimum amount of coconut milk. Evidence for such growth inhibition in certain mature cells will be presented in this paper.

The coconut-milk growth-factor in relation to normal development

The growth-promoting activity designated C.M.F. is most conveniently found in the fluid endosperm of the coconut. This fluid is almost unique in that it forms and persists in quantity while the embryo remains developmentally immature. The question therefore arises how far the activity which is recognizable by the carrot assay procedure is generally present in the nutritional system of immature embryos or to what extent it is peculiar to *Cocos nucifera* in particular or to monocotyledons in general. Also the part played by this growth-promoting activity during the normal development of the fertilized ovule presents a problem which can now be attacked in relation to those plants in which the activity can be demonstrated. Evidence on these lines has been obtained by making extracts from various plants at various developmental stages and by using these in lieu of coconut milk in the standard procedure for growing carrot explants.

Cell proliferation and tumorous growth

In the growth and normal development of tissues in the plant body some cells, e.g. cambia, remain continuously capable of cell division although, as the familiar facts of dormancy and rest period show, the extent to which they exercise this ability may be controlled by other and complex variables. In the majority of cells in the plant body differentiation proceeds concomitantly with the decrease, if not the total suppression, of cell division. However, tumours of one sort or another present notable exceptions in which random proliferation occurs in cells in which division would normally be suppressed. Such tumours, therefore, present another point of attack upon the general problem. The question arises whether the continued random proliferation of tumours is due to, or associated with, the persistence or recrudescence of that kind of growth-promoting activity that may be recognized by the carrot assay system and which has been designated C.M.F. The persistence or recurrence of such activity might be attributable to two different causes. In the one, the effective agent or circumstance would enable the growth-factor itself to recur in cells from which, in the normal course of development, it would have disappeared. In the other, the effective agent or circumstance could be regarded as one which operates through the inhibitory or regulatory substances that normally control the growth-promoting properties and prevent their unrestricted activity in mature cells. Evidence upon these lines has been obtained by the

use of extracts of various plant tumours, in lieu of coconut milk, in the carrot tissue-culture procedure.

All of these lines of investigation clearly converge and overlap; they will now be described in separate papers, of which this is the first, under this general introduction.

Evidence for Growth Inhibitors in Mature Tissues

A considerable literature on inhibitors for germination (Evenari, 1949) and growth (Hemberg, 1951, and references there cited) has developed in recent years. These inhibitors have been considered most in their inter-relations with the auxin system and techniques for the detection of auxin have been prominently used in their investigation. In two areas of plant physiology the role of growth inhibitors has been postulated in ways which are relevant to this investigation. An ether and water soluble growth-inhibiting substance present in the potato-tuber periderm is held to be of significance for the rest-period of the potato (Hemberg, 1949 *a*) and similar substances are supposed to play a similar role in the buds of trees, e.g. *Fraxinus* (Hemberg, 1949 *b*).

It is first necessary to set down the procedures by which growth-inhibitory substances or mechanisms may be detected by the use of the carrot-coconut milk assay system. The general procedures are as follows.

The nutrient and other conditions under which standard explants of carrot root may be accurately and reproducibly grown have been described (Steward, Caplin, and Millar, 1951; Caplin and Steward, 1952). If a large number, say 100 to 150, of such explants are all cut from the same root, one has a uniform population of explants which may be used for a variety of tests. If, to the nutrient medium containing adequate amounts of the coconut-milk growth-factors, extracts are added which do not contain substances which markedly affect the growth-promoting qualities of the coconut milk, little effect on the growth of the carrot explants would be expected. If, on the contrary, there are substances in the extract which suppress the activity of the coconut milk they would also be expected to suppress growth, but there remains, however, one possible source of confusion. The coconut-milk growth-factor system is itself markedly affected by concentration: at too great a concentration growth may be depressed (Caplin and Steward, 1952). Therefore, if added plant extracts suppress growth they might conceivably do so because they contain, not inhibitory substances, but the growth-factor itself and in excess.

A growth inhibition due to too much growth-factor may be distinguished from an inhibition due to a specific inhibitor by experiments in which the diluted plant extract is used to supplement sub-optimal amounts of coconut milk. If the diluted extract in question can *accentuate* coconut milk its activity would be ascribed to the growth-factor: if the diluted extract always *antagonizes* coconut milk its activity is due to an inhibitor. The observation that even growing carrot cultures produce a 'staling-product' which tends to suppress their own growth (Caplin and Steward, 1952) is here recalled.

Tests of this kind show that growth-inhibitory mechanisms are present in a variety of plant systems: these will now be described separately as follows.

Inhibitors of growth in potato tuber

The first observation that mature potato-tuber tissue may contain substances capable of inhibiting the growth of carrot explants in media containing coconut milk was made unexpectedly in the following manner. To test whether a solution containing a wide range of organic nutrients, especially amino-acids, could supplement or replace coconut milk in the growth of carrot tissue an extract of potato tissue was made and added to the otherwise optimum nutrient solution. The then surprising observation was made that this potato extract did not improve the growth of the carrot, in fact it depressed it and even, at an equivalent concentration of 10 g. of potato tissue per 100 ml. of solution, suppressed the growth of the carrot tissue completely.

Experiments were then carried out by making hot-water, filtered, extracts of potato-tuber tissue and adding these to the standard nutrient and coconut-milk solution in such a manner that the final concentration was known in terms of the equivalent of potato tissue added as extract. This was tried using both the tissue freshly cut from the mature tuber and also after thin slices had been exposed to aerated solution to render them more metabolically active (Steward and Preston, 1940). The effect of added potato extract on the growth of carrot in presence of coconut milk and under the standard procedures described (Caplin and Steward, 1952) is shown at Fig. 2, in which the histograms represent the mean fresh weights of five cultures and the standard deviations are indicated.

In view of the fact that potato tissue has such widespread use in microbiology as a general nutrient medium this result was somewhat surprising, the clear implication being that the parenchyma of the tubers in question contained a substance or substances capable of antagonizing the growth-factors contained in coconut milk. Whilst both the metabolically inactive and active tissue contained the inhibitor there was some indication, at the concentration 0·1, that the aerated slices contained somewhat less of the inhibitory activity.

Subsequent attempts to repeat and investigate this effect have produced a variety of results. In some samples of potato tuber the inhibitory effect was slight or non-existent: in others it approached or equalled the striking effect shown in Fig. 2.

A relatively simple way to demonstrate the inhibitory effect of potato tissue on the growth of carrot in coconut-milk culture is as follows: Using the cannula used to cut carrot plugs (Caplin and Steward, 1952), cylinders of potato tuber may be removed and placed in the rotating culture tubes together with the basal medium and coconut milk. The medium and tissue is then autoclaved and the tube is inoculated with a standard carrot explant. Carrying out such an experiment with the potato (variety 'Sebago' from 11° C. storage) and using progressively increasing quantities of tuber tissue, the growth data shown in Fig. 3 were obtained. In this case the inhibitory effect was relatively

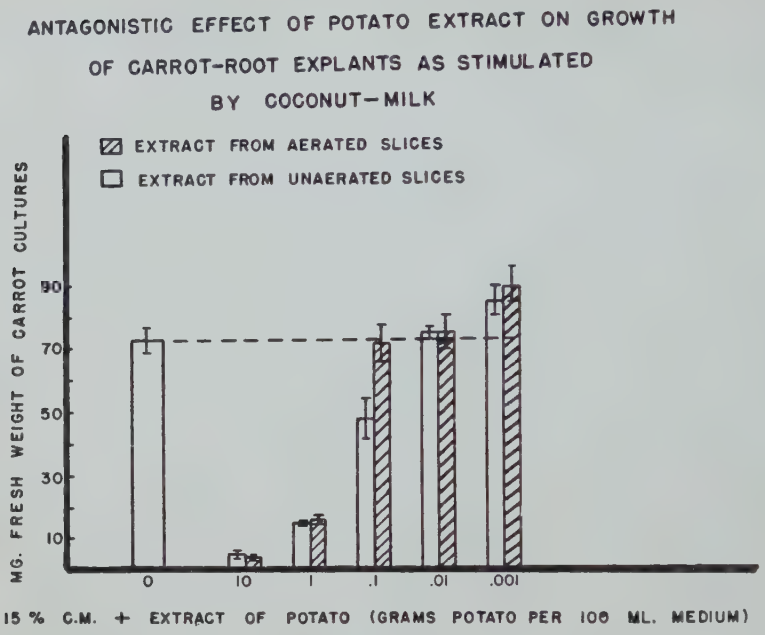
ANTAGONISTIC EFFECT OF POTATO EXTRACT ON GROWTH
OF CARROT-ROOT EXPLANTS AS STIMULATED
BY COCONUT-MILK
EXTRACT FROM AERATED SLICES
EXTRACT FROM UNAERATED SLICES
MG. FRESH WEIGHT OF CARROT CULTURES
90
70
50
30
10
0
10
1
.1
.01
.001
15 % C.M. + EXTRACT OF POTATO (GRAMS POTATO PER 100 ML. MEDIUM)

FIG. 2

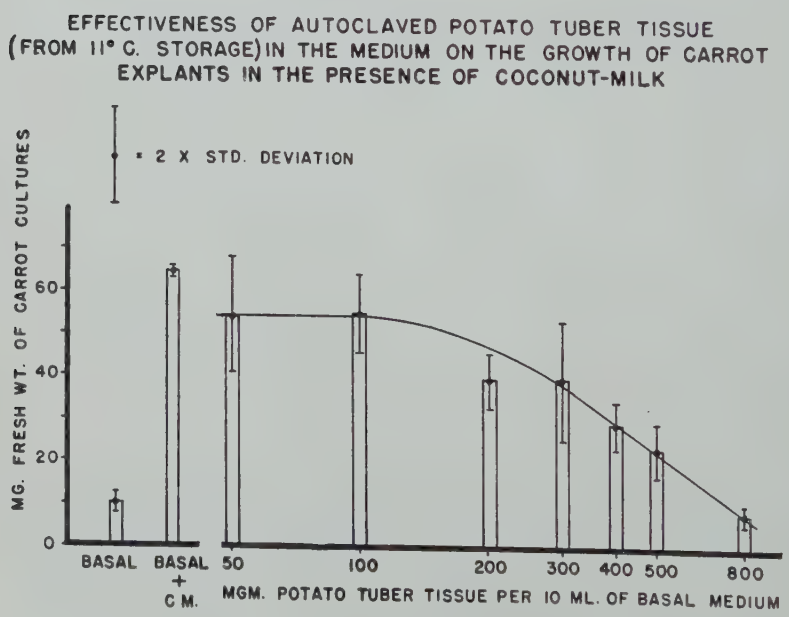
EFFECTIVENESS OF AUTOCLAVED POTATO TUBER TISSUE
(FROM 11° C. STORAGE) IN THE MEDIUM ON THE GROWTH OF CARROT
EXPLANTS IN THE PRESENCE OF COCONUT-MILK
= 2 X STD. DEVIATION
MG. FRESH WT. OF CARROT CULTURES
60
40
20
0
BASAL
BASAL + C.M.
50
100
200
300
400
500
800
MGM. POTATO TUBER TISSUE PER 10 ML. OF BASAL MEDIUM

FIG. 3

weak but was progressive with the amount of added potato-tuber tissue until growth was completely suppressed to the value characteristic of the basal solution without the coconut-milk supplement.

Two factors at least should be considered in relation to the responses in question, for the inhibitory effect of potato extracts upon the growth of carrot stimulated by coconut milk may be related in part to the morphology of the tuber and in part to its developmental history.

Experiments showed that extracts equivalent to 1 per cent. of potato periderm added to the medium markedly reduced the growth of carrot-tissue cultures whereas additions equivalent to 10 per cent. suppressed it completely. Comparisons made between water extracts obtained from the periderm and from the storage parenchyma showed that the inhibitory activity was more pronounced (weight for weight) in the periderm than in the rest of the tuber tissue. This is shown by the data of Table I, which reveals the effect of adding potato extracts at the equivalent of 1 per cent. of original potato tissue (from both freshly dug and chilled tubers) to the nutrient medium. As shown by the growth of explants from two different carrot roots the inhibitory effect was much more evident in the periderm than in the rest of the tuber. Any effect attributable to the chilling was to reduce the inhibitory effect of the potato extract on the growth of the carrot; this latter effect is statistically acceptable in the case of carrot *B*.

TABLE I

Effect of Potato Extracts on Growth of Standard Carrot Explants in Basal Media containing Coconut Milk

	Weight of carrot cultures	
	Carrot *A*	Carrot *B*
Controls	65·4± 8·1	92·6±5·5
Plus 1%* periderm of dormant potato . . .	38·5± 6·5	46·3±4·3
Plus 1% parenchyma of dormant potato . . .	72·7± 4·2	65·4±5·6
Plus 1% periderm of non-dormant potato tuber .	45·6± 6·5	67·7±2·9
Plus 1% parenchyma of non-dormant† potato tuber .	65·6±14·2	87·9±4·6

* Amount of extract added corresponds to 1 g. of tissue per 100 ml. of medium.

† Non-dormant means tubers which had been stored at relatively low temperature.

Searching for the cause of the evident differences in the response of extracts of different potato tubers the only clue is as follows: Extracts from tubers in which the periderm still 'slipped' and which were, therefore, somewhat immature and dormant in the sense that they would not immediately grow if planted, were more prone to yield extracts which inhibit the growth of carrot tissue as stimulated by coconut milk than extracts from old tubers with a storage history.

Thus far there is some indication that the potato-tuber parenchyma, which do not respond by proliferative growth to coconut milk alone, contain some inhibitory mechanism for the growth of carrot tissue. Other evidence has

been reported that potato tissue contains substances which inhibit specific metabolic or other activities. According to Arrequin and Bonner (1949) potato tubers, as normally stored, contain an alcohol-soluble inhibitor of starch-phosphorylase activity; whereas in the tubers stored at low temperature, which would also break dormancy, the phosphorylase inhibitor is supposed to disappear. This suggested that the two effects, i.e. on phosphorylase and on the growth of carrot tissue, might be connected. The alcohol-soluble constituents of potato tubers (variety 'Sebago', obtained immature from Florida) certainly depressed the growth of carrot cultures in a manner similar to the water extracts and the autoclaved tissue: the inhibitory effect on phosphorylase of the same alcohol extracts seemed to be inconclusive so far as these purely qualitative tests went.

Protracted storage at 0° C. of potato tubers var. 'Sebago' (in contrast to tubers stored at 11° C.) suggested that the inhibitory effects of potato tissue, var. 'Sebago', on the growth of carrot explants are reduced by protracted storage at 0° C. in contrast to similar tubers stored at 11° C. (Fig. 3). The inhibitory effect, which on certain 11° C. stored tubers was quite apparent at 200 mg. potato tissue per 10 c.c. of carrot medium (Fig. 3) and was complete at 800 mg., was negligible at the lower concentration of the cold-stored potato, and in some cases it did not appear even at the higher concentration. So far the inhibitory effect of potato-tuber tissue or its extracts might possibly be attributed to the starch which they contain—possibly because of its adsorptiveability. This, however, could hardly explain the greater inhibitory effect of the potato periderm, nor of an onion-bulb extract (Fig. 4) which is to be described below.

These observations therefore lead towards the idea that growth inhibitors for carrot tissue may occur in the potato tuber, the tissues of which fail to proliferate and grow even in favourable media containing coconut milk. The observation, already reported (Steward and Caplin, 1952), that 2,4-D and coconut milk act synergistically to promote the proliferative growth of potato tissue to form a tissue culture is clearly suggestive here. An obvious explanation would be that the 2,4-D exerts its effect through the type of growth-inhibitory mechanism of mature potato cells that has been detected by the carrot assay method. If the potato inhibitor for the coconut milk factor and 2,4-D were related as metabolite and antimetabolite, this would make all these observations intelligible.

In the normal way, therefore, potato cells do not grow in culture for at least two reasons: (i) they lack the growth-factor or factors present in coconut milk; (ii) they possess, in some degree, a growth inhibitor for the C.M.F. In presence of 2,4-D, however, the inhibitor is masked or suppressed so that the potato tissue can respond by proliferative growth to the coconut-milk factor when this is supplied.

Inhibitors of growth in onion-bulb tissue

Again, the discovery that onion tissue may contain substances or mechanisms inhibitory to the growth of carrot as stimulated by coconut milk was obtained

incidentally to experiments made for another purpose. The synergistic effect of 2,4-D and coconut milk on potato tissue (Steward and Caplin, 1951) has already been mentioned. Claims had previously been made (Lucas and Hammer, 1947) and qualified (Spear and Thimann, 1949) that onion juice contained substances that made 2,4-D more effective as a herbicide. There was therefore an obvious possibility that onion juice might be able to *substitute* for coconut milk in the growth of carrot explants.

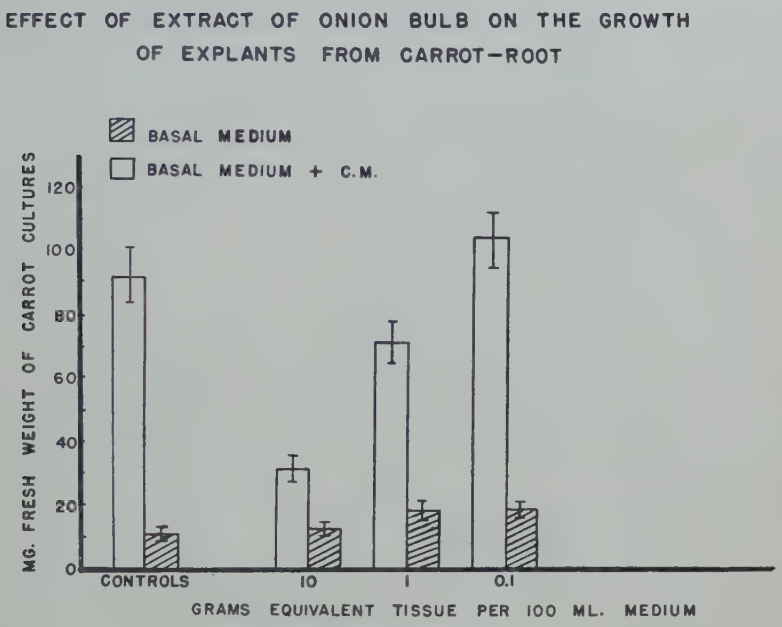

FIG. 4

Hot-water extracts of onion-bulb tissue were made and so added to nutrient media for carrot explants that one could detect whether they contained the growth-factor itself or substances inhibitory to it. For the one purpose (detection of a growth-factor) the onion extract was used in lieu of coconut milk; for the other (detection of inhibitors) the onion extract was added to media containing coconut milk. The data are recorded in terms of the effect of these treatments on the growth of carrot explants under the standard conditions of these experiments and are shown in Fig. 4. The implication is clear, namely, (i) onion extract cannot effectively substitute for coconut milk in the growth of carrot tissue; (ii) onion extract acts as an inhibitor to the growth of carrot as stimulated by coconut milk.

Again we see that a mature tissue, which in this case has not yet been successfully cultured, contains an inhibitor for the carrot–coconut milk system.

Inhibitors of growth in dormant buds

Further evidence has been obtained of the regulatory role which growth-factors detectable by the carrot assay system and their inhibitors may play. This flows from a study of the physiology of bud dormancy made by Dr. B. Pollock, working under the direction of one of us (F. C. S.). Extracts of dormant and non-dormant buds were tested by the carrot assay technique for their growth-promoting or growth-inhibitory activity by Pollock during

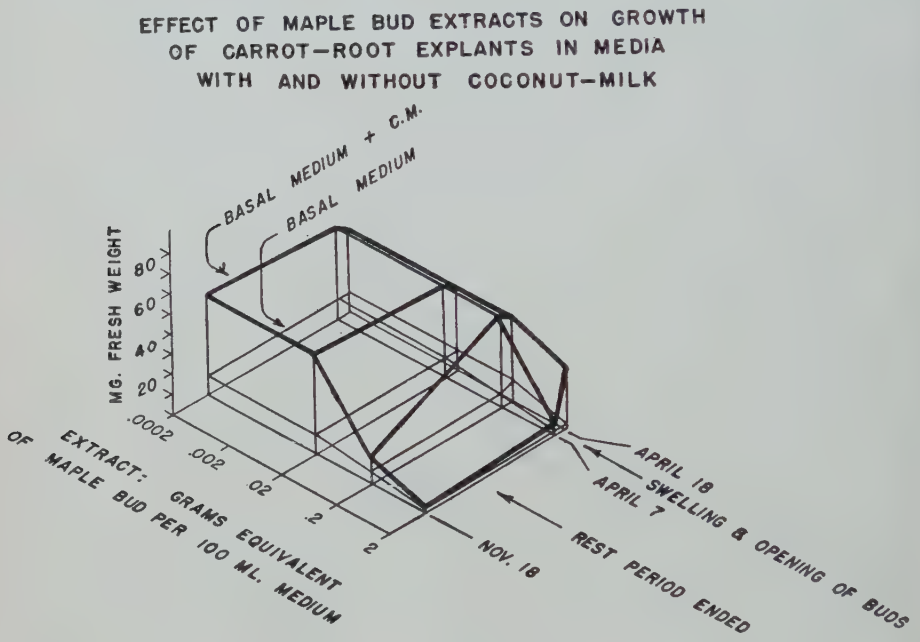

FIG. 5

the winter of 1948–9: Pollock has also summarized the data obtained (1949). The effective data are presented in Fig. 5, which shows the following results.

i. Extracts of maple buds never show positive growth-promoting activity which may replace coconut milk in the growth of carrot.
ii. Dormant maple buds do, however, yield extracts which have inhibitory activity towards the growth of carrot tissue under our standard procedure.
iii. As the rest period advances and after winter chilling there comes a point beyond which the inhibitory activity is much reduced; it then only becomes evident at much higher concentrations of the maple extract than hitherto.
iv. When the buds begin to swell the inhibitory substance for the growth of carrot seems to disappear almost entirely.

Again there is the evident implication that in this type of tissue (buds)

specific substances or mechanisms may exist which inhibit, at considerable dilution (order of 0·02 g./100 ml. of solution), the action of the coconut-milk growth-factor on carrot.

General Discussion

It will be clear, therefore, that specific substances or mechanisms which inhibit the growth-promoting action of coconut milk on carrot tissue can be demonstrated in various plants and tissues. Specifically this has been observed for potato tissue, especially the periderm and more particularly for the dormant, or even the immature tubers; for extracts of onion bulb tissue and maple buds, especially when the latter are in their dormant state. In all these cases the inhibitory mechanism has been demonstrable at relatively low concentrations. The diversity of the materials tested, the evident occurrence of the inhibitor in relatively dormant, physiologically inactive, or storage tissue, and the relation between the inhibitor and the physiological state of the tissue, all suggests that the inhibitor and/or the growth-factor with which it interrelates may be part of a more general regulatory mechanism than the carrot assay method would alone imply.

In fact experience has shown that extracts of many mature plant tissues will completely suppress the growth of carrot in coconut milk if they are added in sufficient concentration. When the water-soluble constituents are added in concentrations of the order of the equivalent of 5 to 10 g. of tissue to 100 ml. of solution, the growth of the carrot is commonly reduced or suppressed. In this paper attention has been more particularly drawn to cases in which the inhibitory effect is evident at even greater dilution. However, the prevalence of this inhibitory effect in extracts from mature cells and tissues suggests that parenchyma, which usually contain the main organic nutrients necessary for growth (i.e. sugars, amino and organic acids, &c.), may often fail to grow either because they lack specific growth-factors for cell proliferation or they are prevented from responding to them, even when supplied in the form of coconut milk, because they also contain inhibitors of the kind which are here demonstrated by the use of the carrot assay procedure. On these lines the effect of 2,4-D on the tissue of the potato tuber in presence of coconut milk could be explained by the fact that it releases the potato tissue from the control of the growth inhibitor so that it can respond to the coconut-milk factor when this is supplied.

Summary

1. The growth of explants taken from various plant storage organs in a nutrient medium containing coconut milk has been described for conditions that are conducive to the growth of carrot tissue. Some tissues, e.g. from Jerusalem artichoke (*Helianthus tuberosus* L.), respond by growth as vigorous as in the case of carrot root: others, sweet potato (*Ipoemea Batatas* Lam.), parsnip (*Pastinaca sativa* L.), and white turnip (*Brassica campestris* var. *rapa*) respond in much less and in varying degree.

2. Growth under controlled conditions of standard explants of carrot phloem in media containing coconut milk has been used to detect inhibitors of growth: the necessary techniques are described.

3. Evidence for such inhibitory substances or mechanisms has been found in (i) potato tubers, (ii) onion bulbs, and (iii) maple buds.

4. The evidence is that the inhibitory substance in the potato is extractable by water and alcohol and is (i) more abundant in the periderm than the parenchyma; (ii) more evident in tissue from dormant (unchilled) than non-dormant (chilled) tubers.

5. It appears that the effect of the inhibitor on the potato tuber tissue is counteracted by 2,4-D which permits this tissue to grow in presence of coconut milk.

6. Extracts from onion bulbs are markedly inhibitory to the growth of carrot tissue and coconut milk.

7. Extracts from dormant maple-buds are inhibitory to the growth of carrot tissue in coconut-milk media, but this inhibition appears to be reduced as the dormancy expires and virtually disappears when the buds swell.

8. It is suggested that a factor for cell proliferation, such as that present in coconut milk, together with the substances which may inhibit its activity can be visualized as parts of a regulatory mechanism of growth that is more widespread than the carrot assay would alone imply. Parenchyma may fail to proliferate in nutrient media for two different reasons: (i) it may lack the essential growth-factors; (ii) it may contain growth inhibitors detectable by the carrot assay procedure.

Acknowledgements

Much of the work here described was carried out in the Department of Botany, University of Rochester, Rochester, N.Y. It was originally made possible by grants to one of us (F. C. S.) from the National Cancer Institute, National Institutes of Health, Washington, D.C., U.S.A., and in its continuation has been supported by grants from the same source to each of the authors, i.e. at Cornell University and the University of Rochester, respectively. Our gratitude is here expressed for this help.

Miss Mary Case served as research assistant and carried out most of the manipulations requiring aseptic technique: her help is gratefully acknowledged.

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Investigations on Growth and Metabolism of Plant Cells

IV. Evidence on the Role of the Coconut-Milk Factor in Development

BY

F. C. STEWARD[1]

AND

S. M. CAPLIN[2]

With five Figures in the Text

ABSTRACT

By the carrot-assay method it has been shown that the watery endosperm of coconut contains the growth-promoting coconut-milk factor at all stages of development. Some activity is shown by the parts of the immature embryo but not by the solid endosperm.

Sources of analogous activity are in the endosperm of *Zea mays* in the milk stage, the gelatinous content of immature fruits of *Juglans regia*, and the young gametophyte of *Ginkgo biloba*. The data for other cases examined suggest that the material develops best in nutritive tissues associated with delayed embryo development.

MORPHOLOGICALLY the fluid known as coconut milk represents the more fluid portion of the endosperm of *Cocos nucifera*. The single coconut embryo, situated beneath the single functional micropyle, is relatively immature and is embedded in the more solid portion of the endosperm. On germination the embryo produces a remarkable cotyledonary outgrowth which penetrates into the central cavity and absorbs the nutrients from the milky fluid to permit the very rapid development of the embryo that then ensues. The morphology of the coconut was described by Kirkwood and Gies (1902). The interest which attaches to the coconut milk derives from its role as the nutritive fluid for the immature embryo and its subsequent rapid development. The well-known application of coconut milk in the culturing of embryos of *Datura* by Van Overbeek, Conklin, and Blakeslee (1941) and of *Ginkgo*, *Lilium*, and a variety of dicotyledonous embryos by Tukey (1944) follows naturally from the appreciation of its morphological nature and nutritional role in the coconut plant.

The discovery that coconut milk is effective in obtaining the most rapid growth of tissue cultures of other angiosperms (Caplin and Steward, 1948,

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[Annals of Botany, N.S. Vol. XVI, No. 64, October, 1952.]

confirmed by Duhamet, 1950; Gautheret and Duhamet, 1950; Morel and Wetmore, 1950; Nickell, 1950) leads to the following obvious problems:

1. Is the activity of the coconut milk in any way a function of its stage of development or of the degree of maturity of the fruit?
2. Is the activity that is demonstrable in the coconut unique to that plant or may it be found in other comparable morphological situations?
3. Is the activity demonstrable elsewhere in the plant body or is it peculiar to endosperm?

The evidence which bears upon these questions and which has been obtained by testing a variety of plants and extracts by the carrot-assay procedure will now be presented.

Coconut-milk Factor in Relation to the Development of the Fruit

Ideally this study should have been made with controlled pollination so that the development of the fruits in question could be related to the stages of pollination and fertilization. This was, however, not possible. Entire fruits, with their fibrous outer husk attached, were collected in Florida by the courtesy of the Dade County Parks, Miami, Fla., and shipped to the laboratory in Rochester, New York, for examination. The samples examined covered the entire range of fruit sizes that could be collected from the palms at one time. On arrival the weight and length of each whole fruit was obtained, the nuts dissected and their lengths determined, and the volume and appearance of the milky fluid was recorded. The status of the more solid endosperm was also recorded. The range covered by these samples may be appreciated since the volume of fluid extracted from single fruits ranged from 3 to 700 ml. All of these sampled fruits had developed after fertilization and their size is to be taken as the criterion of their age and stage of development. The details on these samples are given in Table I.

The extracted fluid was first frozen and stored at —20° C. prior to assay. One series of assays covered all the samples using carrot explants from a single carrot. In each case the coconut milk was diluted to 15 per cent. This series suffices to test for the presence of the growth-promoting activity at all ages (sizes), but it does not allow for the possibility that in some of the stages the concentration of the growth-factor might be such that 15 per cent. by volume in the nutrient medium might not be the most suitable concentration for the assay. To guard against this contingency, three widely separated size stages were selected and at these stages carrot assays were performed using the coconut milk at concentrations ranging from 1 to 40 per cent. by volume.

Data are presented in Fig. 1 for the first of these series of assays (i.e. relating activity at 15 per cent. by volume to the size of the husked nut). It is apparent that the fluid endosperm is active at all stages in its development; and allowing for possible variability, no significant effect of age on activity could be seen. (The apparent tendency of these data to fall into two groups, though suggestive

TABLE I

Arbitrary size No.	Overall length of fruit (mm.).	Length minus husk.	Vol. of extracted fluid.	Remarks	
				Fluid endosperm.	Solid endosperm.
1	68	34	2·8	Brown with some flocculent precipitate.	Husk white at base.
2	67	31	3·5	Brown with some flocculent precipitate.	Husk brown at base. Ovary wall soft.
3	87	51	17·5	Clear, almost colourless, very little precipitate.	Ovary wall soft.
4	89	54	17·4	Very light brown in colour, slight precipitate.	Ovary wall soft.
5	88	56	15·3	Light brown with considerable precipitate.	Ovary wall soft.
6	116	73	37	Quite opalescent, some precipitate.	Ovary wall soft.
7	116	91	95	Clear and colourless with slight opalescence.	Ovary wall soft.
8	128	83	105	Water-clear.	Ovary wall soft.
9	134	94	235	Slightly opalescent, some flocculent precipitate.	Ovary wall soft; solid endosperm less than 1 mm. thick.
10	146	110	215	Opalescent; slight flocculent precipitate.	Ovary wall soft; solid endosperm about 1·8 mm. thick.
11	136	85	240	Water-clear; very little turbidity.	Ovary wall soft; very thin solid endosperm layer.
12	151	96	350	Clear; turbidity almost negligible.	Ovary wall soft; solid endosperm layer about 1·0 to 1·4 mm.
13	153	94	385	Very clear.	Ovary wall thin; solid endosperm layer less than 1·0 mm.
14	186	116	330	Opalescent.	Slimy layer forming at basal end.
15	186	117	575	—	Ovary wall thin; solid layer conspicuous.
16	199	125	650	Practically water-clear.	Solid endosperm layer 4·4 mm., firm.
17	207	123	560	Slight turbidity.	—
18	245	145	540	Opalescent with suspended matter.	Prominent solid endosperm, 7·8 mm. thick at basal end.
19	242	138	710	Opalescent.	—
20	244	158	600	Opalescent.	Solid endosperm 7·7 mm. thick.

of two different sources (e.g. of two different palms), cannot be easily explained as the fruits were mixed after collection.)

From the dilution series (Sizes Nos. 4, 8, and 18) it was apparent that 15 per cent. by volume of coconut milk in the nutrient solution was approximately the optimum concentration: only in the case of the oldest nuts (No. 18) did an excess of coconut milk markedly *depress* the growth of the carrot. Thus the fluid endosperm of the coconut is remarkably constant in its content of the growth-factor even over a wide range of developmental stages.

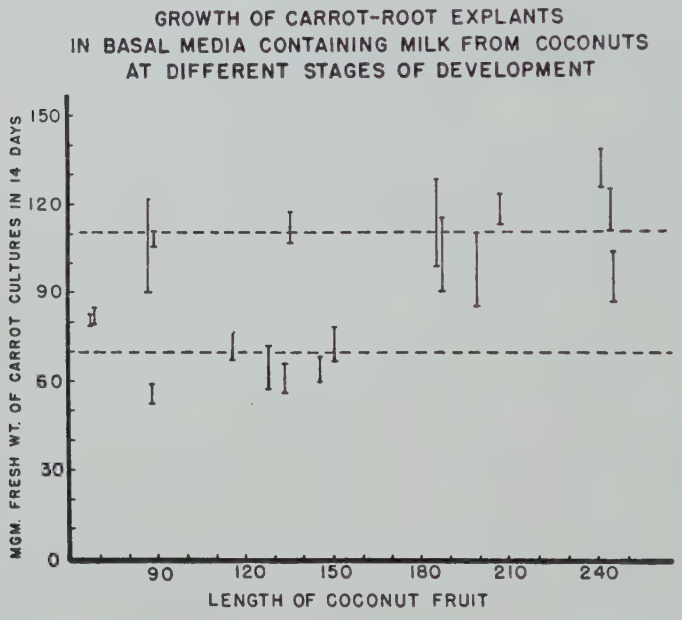

FIG. 1

At all the developmental stages recorded above water extracts of the more solid endosperm of the coconut were inactive in the carrot-assay procedure. In other studies by E. M. Shantz and one of us (F. C. S.) some activity has been obtained by the use of the solid endosperm digested by proteolytic enzymes. It is not suggested, however, that this is greater than could be attributable to other protein hydrolysates (e.g. casein) alone.

EFFECT OF GERMINATION ON THE COCONUT-MILK FACTOR

The germination of the mature coconut may be delayed for a relatively long period. However, in any suitable environment fruits may be collected showing the young shoot and root which have emerged from the 'husk'. If such fruits are cut across they show the remarkable development of a spongy cotyledonary tissue which fills the entire cavity originally occupied by the fluid endosperm and which (see Table I) may be up to 700 ml. in volume.

This spongy tissue has a differentiated and presumably absorptive surface layer. The progressive changes which accompany the maturation and germination of the embryo in such fruits as Nos. 19 and 20 of Table I and their effects on the composition and growth-promoting qualities of the originally fluid endosperm would clearly repay for detailed investigation. This, however, has not yet been possible, but it is of interest to report the examination of germinated coconuts at the stage indicated above. In the nuts examined the seedling leaves and roots had emerged. The 'cotyledonary' tissue filled the entire endosperm cavity. Samples were taken of the spongy cotyledonary tissue, of its absorptive surface, and of the shoot tissue of the young seedling.

In assaying these extracts use was made of a 1951 stock of carrots (var. 'Chantenay', grown in Ithaca, N Y.) which was known to contain roots which, in varying degrees, responded to casein hydrolysate as well as to whole coconut milk. Tests were therefore carried out using casein hydrolysate, coconut extracts, and both of these in combination as supplements to the basal medium. The results of the tests and appropriate controls will be evident from Table II. These were made by making water extracts (1 : 10) and adding these to the medium in the equivalent concentrations indicated and in lieu of coconut milk. From the table it is clear that the spongy tissue of the cotyledon, though part of the embryo, retains some of the activity of the original milk. The examination of the embryo shoot is particularly interesting in view of the discussion to follow in a later paper: this discussion will deal with the growth-factor in relation to mature tissues and to tumours. The tissues of the rapidly growing embryo absorb the growth-factor from the 'milk' so that activity may be demonstrated, especially if casein hydrolysate is also supplied, in both the cotyledon and the young shoot, or plumule.

TABLE II

The Growth of Carrot Explants during 21 Days in Media containing Extracts from Various Parts of Germinated Coconut

	Casein hydrolysate added.	
	None. Final wt. of carrot cultures (mg.).	0·05%. Final wt. of carrot cultures (mg.).
Basal medium only . . .	13·2± 1·7	48·0± 8·4
,, ,, +10% coconut milk	176·2±21·2	206·3± 8·5
,, ,, +extract of:		
Interior of cotyledon* .	62·4±13·2	137·7±21·6
Surface of cotyledon .	40·0± 8·4	48·9± 3·8
Shoot	25·7± 3·0	67·0± 9·0

* Spongy interior of cotyledon 17·8% dry wt.; absorptive surface 31·8%; embryo shoot 23·0%.

'COCONUT-MILK FACTOR' FROM OTHER MONOCOTYLEDON SOURCES

Thus far there is a strong presumption that the activity designated coconut-milk factor is peculiarly of endosperm origin. The question arises whether it may be found in other similar situations in monocotyledons.

The first possibility tried was the case of *Zea*, which is well known for its so-called 'milk stage'. In this stage the endosperm, though cellular, has very thin walls (Randolph, 1936), and it is only later that starch deposition proceeds. Collections were made of developing *Zea* grains in each of two seasons. In the first (1948) we owe the samples to the co-operation of Dr. Watson, then of the Dept. of Agronomy, University of Illinois. The immature grains were lyophilized and the lyophilized product was subsequently extracted with hot

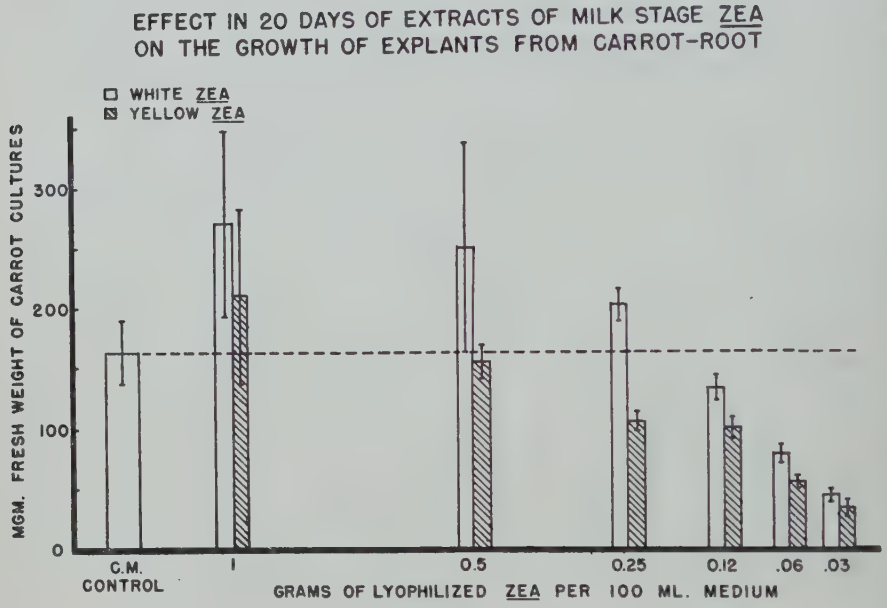

Fig. 2

water and the water extract was used in lieu of coconut milk in the carrot-assay procedure. In the second (1949) the procedure was repeated but the lyophilized samples were collected at known dates following the shedding of the pollen. The results are shown in the two Figs. 2 and 3. The first shows that water extracts made from two varieties (white and yellow) of *Zea* sampled in the milk stage were able to yield extracts which were as potent in stimulating the growth of carrot as coconut milk, in the sense that at the proper concentration (extract equivalent to 1 g. of lyophylized corn per 100 ml. of medium) the carrot grew as well as with coconut milk alone at the optimal concentration. In some cases it may have been slightly better, but probably not more so than coconut milk plus the additive effects that have been observed with casein hydrolysate, malt extract, &c. This clearly indicates that *Zea* in the 'milk stage' and coconut milk at all stages of development are comparable in the sense that they both contain the particular growth-factor or factors in question.

The data of Fig. 3 show that for *Zea*, in marked contrast to the coconut, the activity of the preparation is related to the stage of development. It was

an obvious and easy experiment to test mature grains of *Zea*. All such tests show that the activity of such extracts for the growth of carrot is small if they are made from mature grains: even at the optimum concentration the growth of the carrot rarely exceeds 20 per cent. of that on coconut milk. By sampling the developing grains at different stages of development after pollination the data of Fig. 3 were obtained. These showed high activity at 2 weeks after pollination followed by a decline to a low value after 8 weeks. Relating growth-

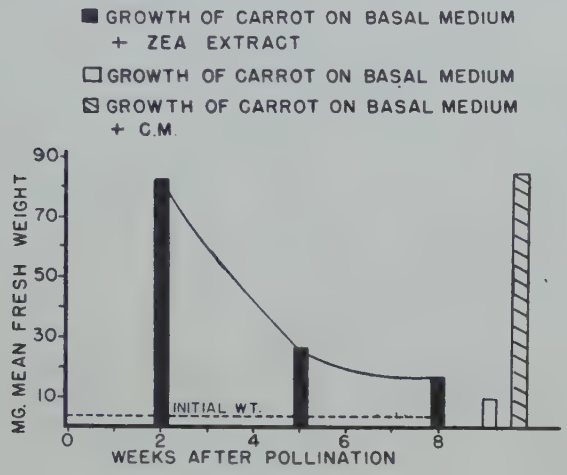

FIG. 3

promoting activity to the weight of dissected grains it can also be shown that the growth-promoting activity for carrot increases after pollination and then declines. This is shown by the growth promoted in comparable carrot cultures by samples of grains of field corn which had the weights shown below. The *Zea* was tested at amounts of 1·2, 2·5, and 5 mg. per 100 ml. of nutrient solution: the data are shown in Table III.

TABLE III

Effect of Age of Dissected Field Corn (Zea mays) Grains on their Ability to promote the Growth of Carrot Explants in Sterile Culture

Conc. in g. per 100 ml. of nutrient.	1·2			2·5			5·0		
Grain size in mg. .	12	111	161	12	111	161	12	111	161
Wt. of carrot culture grown per culture of 10 ml. . .	8·2	50·0	34·9	4·6	38·4	17·8	2·5	12·7	12·2

Prior to pollination the grains weigh 12 mg. each and their activity towards carrot is clearly very small. (Similarly extracts of *Zea* pollen were totally inactive towards the carrot-assay test.) These results on pollen and on ovules

prior to pollination were repeatedly obtained. The grains at 111 mg. (some time after pollination and approximately $\frac{1}{4}$ of maximum size) were quite active and those at 161 mg. (approximately $\frac{1}{3}$ grown towards full size) though active had considerably less activity. Therefore in all essential respects these data extend and confirm, for a different season and variety, the data of Fig. 3.

Some further tests have been made on stored samples of *Zea* fruits to ascertain whether the dried or lyophilized preparations were the more active and whether the decline in activity with development is in any way concerned with a 'casein factor', which some carrots require for maximum growth, or only to the 'coconut-milk factor'.

Some evidence has been obtained to suggest that the growth-promoting activity is retained somewhat better in lyophilized than heat-dried preparations and also that the activity of the latter, more than the former, is increased by casein hydrolysate. Some evidence suggests that the decline in the activity late in the developmental period tends to be somewhat less if the assays are performed in presence of casein hydrolysate.

What these additional experiments seem to mean is as follows.

The growth-promoting effects demonstrable with whole coconut milk are replaceable by extracts of immature fruits of *Zea* and the activity of these extracts changes during development. The activity in question is probably not due to a single substance but rather to one main heat-stable active principle, or system (as in coconut milk), with interactions with other substances that are replaceable by casein hydrolysate, that tend to be destroyed on heat-drying and that tend to disappear in the maturing grain. Whether the effects attributable to casein hydrolysate are due to some specific nitrogen compound that it furnishes or are due to its ability to form complexes with heavy metals in the nutrient solution is, at this stage, an open question.

Activity and Stage of Development

The clear implication of the results illustrated above is as follows.

The activity which has been designated 'coconut-milk factor' is not conspicuously present in the ovule and embryo-sac prior to fertilization. Following fertilization and as part of the events which surround the formation of endosperm, the activity in question increases rapidly. In the case of *Cocos* the activity persists until the fruit reaches its mature size. In the case of *Zea*, in which the embryo reaches early in the development of the fruit a much more advanced stage than in the case of the coconut, the activity reaches a peak and then, after a relatively short period, begins to decline. In the outcome the growth-promoting activity (C.M.F.) of mature *Zea* grains is either small or absent. This is in contrast to the activity of the fully developed but ungerminated coconut with its *immature* embryo, in which the growth-promoting activity remains at its high level. That the contrasted behaviour of *Zea* and the coconut is somehow to be related to the differentiation of the embryo seems almost certain. It appears that when the growth of the embryo

becomes established and organized the factor for random proliferation declines (*Cocos*), or disappears (*Zea*), or is brought under regulatory control.

Other Possible Sources of the Growth-factor

In Monocotyledons. The question may well be asked, Do other monocotyledons behave similarly? Although some extensive samplings have been made on other cereals and grasses it has not been possible to demonstrate activity as great as that to be found in immature *Zea*. The figures actually recorded for developing wheat grains showed an activity of approximately 25 per cent. of that in coconut milk when the extract was used at a concentration equivalent to 5 g. of immature wheat grains to 100 ml. of nutrient medium. Comparable extracts from immature barley did not exceed 20 per cent. of the activity attainable in coconut milk at its optimum concentration. Extracts of fertilized oat ovaries had their maximum observed activity when they weighed 10 mg. and then the water extract from them was about one-third as effective as coconut milk, whereas ovaries before pollination had no significant activity. When the ovaries had reached 25 mg. they again had no growth-promoting activity for carrot explants. Experiments made by adding extracts of oat ovaries to the basal medium plus coconut milk did not furnish any conclusive evidence that the fruit accumulates an inhibitor as it grows. The explanation probably is that the development of wheat, barley, rye, &c., following fertilization is so rapid that an embryo and its accompanying endosperm reaches in a very few days a stage of development which is comparable to that of *Zea* which is much older as measured in time. In other words, in these plants the stage after fertilization in which the growth-factor may be present, with activity comparable to that in the coconut, may be so transient that much more frequent sampling and precise methods of isolation would be necessary to detect it: to the present it has not been possible to do this.

In Dicotyledons. If the activity (C.M.F.) detected by the carrot-assay method is to be attributed to the early stages in development of embryos, and particularly in cases where these are nourished by materials from the nucellus or embryo-sac, which pass through a very watery or gelatinous state, then it would seem possible to detect this activity in a dicotyledon source.

Albuminous seeds or fruits with persistent endosperm might then be more favourable examples than exalbuminous ones. For this reason seeds of *Ricinus* were sampled at stages during their development and developing fruits of *Fagopyrum* were also collected and assayed. Without recording the data in detail it may be stated that no activity comparable to that of coconut milk in the carrot-assay test was obtained in either of these cases. *Ricinus* extracts were made from seeds which ranged in size from as little as 17 mg. to as much as 500 mg. In view of the results obtained with coconut milk and extracts of *Zea* in the milk stage, the completely negative results obtained with *Ricinus* are somewhat surprising. At 0·1 per cent. or more (equivalent of 0·1 g. of castor bean to 100 ml. of medium) the castor bean showed very markedly the

presence of inhibitors of growth (see Fig. 4 *a*). Similar results were obtained with immature *Fagopyrum* fruits, namely: (i) An extract of the immature fruit could not substitute for coconut milk in the growth of the carrot. (ii) The extracts contained substances markedly inhibitory to the growth of carrot as stimulated by coconut milk (see Fig. 4 *b*, and cf. Steward and Caplin, 1952).

Since these albuminous dicotyledons yielded no activity comparable to that in *Zea* and in the coconut, it may not be so surprising that the first

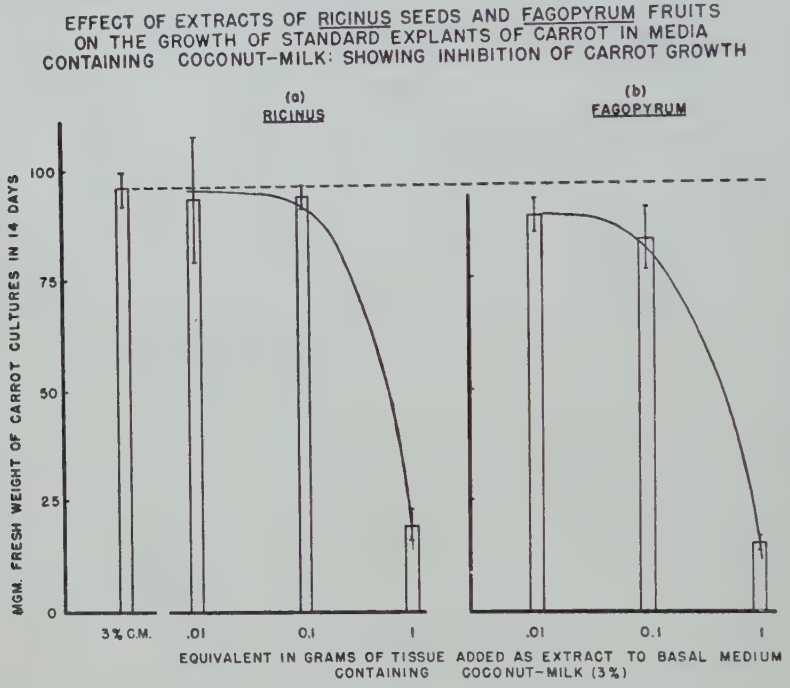

FIG. 4

attempts to demonstrate the growth-promoting activity in developing exalbuminous seeds also failed. Extensive attempts were made using developmental series of legumes (*Vicia faba*, *Phaseolus vulgaris*) and of apple (*Malus*), but these were all negative. For this reason greater interest attaches to the following case where positive results were obtained.

Searching for a dicotyledon source of the growth-promoting activity so conspicuous in coconut milk it seemed that one should seek a plant in which the development of the embryo was delayed and in which the nutrient material from which it was eventually to be nourished accumulated in advance of the embryo's need. We are indebted to Professor L. H. MacDaniels of Cornell University for the suggestion that the fruit of the walnut (*Juglans regia*) is of this type. In this the growth of the cotyledons into a mass of nutrient material, which is first liquid then jelly-like, is slow and, in fact, the ability of the nuts to mature in the season of their development may be limited by the slow rate

of growth of the cotyledons. Accordingly water extracts were made of walnut fruits in which the growth of the cotyledons, although well advanced, was by no means complete. Again the extracts were assayed, by adding them, in lieu of coconut milk, to media in which the standard carrot explants were grown. Since the stock of carrots then in use was known to contain some roots which were responsive to casein hydrolysate, additively to coconut milk, the tests were made with and without the addition of casein hydrolysate to the basal

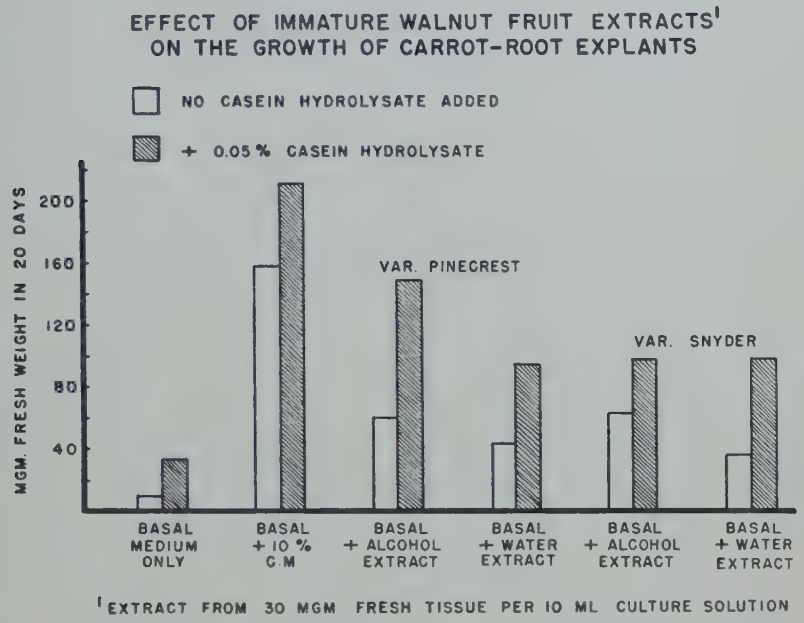

FIG. 5

nutrient medium. The data are contained in Fig. 5, from which it is clearly evident that this dicotyledonous source furnishes some evidence of the same kind of growth-promoting activity as coconut milk. These particular samples were collected relatively late in the season when the cotyledons were somewhat over half grown. It is possible, therefore, that earlier samples would have been even more active.

In Gymnosperms. The growth-promoting activity (C.M.F.) which is assayed by the carrot-assay method has now been shown to be present in certain endosperms and certain cotyledons which grow at the expense of these endosperms. The question therefore arises whether in its origin the growth-factor is peculiar to tissue which has the unique cytological features of endosperms, or whether the emphasis should rather be placed upon the peculiar nutritional role which these endosperms play. To test this a further examination was made selecting *Ginkgo biloba*, the only example readily available of a large gymnosperm female gametophyte. This was selected because it is a haploid tissue, in contrast to the angiosperm endosperm, but it has

a nutritional role somewhat comparable to that of endosperm in the angiosperms.

From the time when the ovules were large enough to dissect (July) collections were made at monthly intervals until October. The ovules produced in the previous year, which normally fall to the ground, were also collected. From these the female gametophytes were dissected and extracted with hot water (20 g. of tissue to 100 ml. of solution). These extracts, diluted so that the final nutrient contained the equivalent of 5 g. per 100 ml., were used in lieu of coconut milk in the carrot-assay procedure. The result was that gametophytes dissected early in the season (July 8) yielded extracts which were 62 per cent. as effective as optimal concentrations of coconut milk. Extracts from the later samples of gametophytes declined rapidly in their activity (gametophytes which had over-wintered, and presumably after the embryo had developed, were even less active). This single set of observations, made only in one season, suffices to indicate that it is possible to obtain this kind of growth-promoting activity from a gymnosperm source. A full understanding, however, of these relationships clearly requires much further work.

Discussion

The so-called coconut-milk growth-factor, or its equivalent, as detected by carrot assay, has therefore been found in four conspicuous places in the plant kingdom. First in coconut milk itself, second in the immature grains of *Zea mays*, third in the ovules of walnut (*Juglans*) with immature embryos, and fourth in the developing gametophyte of *Ginkgo biloba*. *The feature that these varied sources seem to have in common is their ability to grow at the expense of, or to be nourished by, nucellar tissue and to store nutrients for an eventual and very specific nutritive function.* In three of the cases (*Cocos*, *Zea*, *Juglans*) it is an immature embryo that eventually benefits from the growth-factor, which is to be found in a relatively watery and later gelatinous endosperm that grows at the expense of the nucellus. In the fourth case (*Ginkgo*) the growth-factor was found in a female gametophyte, also nourished by a nucellus, and itself furnishing nutrients for archegonia and, later, embryos. In some endosperms the duration of the growth-promoting activity is brief (*Zea*); in others more protracted (*Cocos*). In the case of *Ginkgo* the duration of the activity of the gametophyte also appears to be brief: though it has not yet been possible to specify any relationship to the development of the archegonia.

In general, however, the conditions that are conducive to the accumulation of this growth-factor seem to involve the relatively delayed growth of the structure to be nourished (*e.g. the embryo*) *and the relatively precocious development of the nutritive material* (*e.g. endosperm*) *at the expense of the nucellus.*

Summary

1. Using the standardized carrot-assay method a survey has been made of the relationship between the occurrence of the growth-factor in the coconut and its development.

2. From pollination and fertilization onwards the watery endosperm is always active in the growth substances even until the nut is fully mature. The more solid endosperm that develops relatively late in the growth of the nut is inactive. During this entire period the embryo is immature and unorganized.

3. When germination eventually occurs the cotyledonary growth which replaces the liquid endosperm also has growth-promoting activity and this may also extend somewhat into the shoot of the young embryo.

4. The growth-promoting activity which occurs in coconut milk can also be found in immature grains of *Zea mays* when the endosperm is at the 'milk stage'. The activity appears after pollination (both pollen and unfertilized ovules are inactive), attains its maximum activity some 2 weeks after pollination and, thereafter, declines so that it cannot be demonstrated in the native grain.

5. In other monocotyledonous fruits which have been tested (oats, wheat, rye, barley) the relative development of embryo and endosperm seems to be such that the period of maximum activity of the growth-factor is short.

6. It has not been possible to demonstrate the coconut-milk growth-factor in a variety of immature albuminous and ex-albuminous dicotyledonous seeds and fruits. In the albuminous seed (*Ricinus*) and fruit (*Fagopyrum*) examined the later development is accompanied by the marked accumulation of an inhibitor of growth in the carrot–coconut milk system. A positive demonstration was, however, possible in the case of immature fruits of walnut (*Juglans regia*). In this case the endosperm is somewhat comparable to the coconut in that it passes through a watery and later gelatinous stage and it develops somewhat precociously relative to the embryo, which later grows slowly at its expense.

7. A positive detection of the growth-factor in the young gametophyte of *Ginkgo biloba* was made and its significance noted.

8. The conditions conducive to the accumulation of the growth-factor, which may be recognized in aqueous extracts by its action upon carrot explants, seem to be as follows. The growth-factor is to be found in nutritive tissues which develop at the expense of the nucellus and it accumulates under such conditions that the development of the eventual structure to be nourished (e.g. an embryo) is relatively delayed, while the nutritive tissue (e.g. endosperm) develops somewhat precociously.

Acknowledgements

The work here described was largely carried out at the University of Rochester, though it was continued by one of us (F. C. S.) at Cornell University. The work in both laboratories has been made possible by grants from the National Cancer Institute, National Institutes of Health, Washington, D.C., U.S.A.

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Contribution to the Embryology of Indian Euphorbiaceae

I. *Euphorbia rothiana* Spreng.

BY

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With two Figures in the Text

ABSTRACT

The paper presents an account of the embryology of *Euphorbia rothiana* Spreng.

INTRODUCTION

THE Euphorbiaceae is one of the largest families of dicotyledonous angiosperms and is of special interest due to a considerable variance in its embryological pattern. The members of the family are, in general, mesophytes and xerophytes and are well represented in India.

Baillon (1858) first attempted a morphological description of the group and Lyon (1898) gave the first definite account of the embryology of *Euphorbia corollata*. In the several species which have been investigated, the development of the microsporangium and male gametophyte are of the same general type, although D'Amato (1947) has pointed out a difference in the number of nuclei in the microspore at the time of pollination.

Schmidt (1907) and Modilewski (1910) have reported more than one megaspore mother cell in their preparations, but most of the later investigations do not confirm this. D'Amato (1939) has distinguished four different types of embryo-sac development in *Euphorbia* and these have been tabulated by Maheshwari (1942). It appears, however, that the normal eight-nucleate type is of commonest occurrence. The *Allium*-type was described in *E. mauritanica* by Ventura (1933), the *Penaea*-type in *E. procera* and *E. palustris* by Modilewski (1909, 1911), and the *Fritillaria*-type in *E. dulcis* by Carano (1926). D'Amato (1939) has described normal embryo-sacs in *E. paralias*, *E. falcata*, and *E. pubescens*, and *Scilla*-type in *E. characias* and *E. amygdaloides*. Normal types were again described by him (1947) in *E. nutans*, *E. exigua*, *E. dendroides*, *E. segetalis*, *E. terracina*, *E. prostrata*, and *E. bojeri*, and *Scilla*-type in *E. lagascae*.

Lyon (1898) reported ephemeral antipodals in *E. corollata*, but in the several species figured by D'Amato this does not appear to be the case.

[Annals of Botany, N.S. Vol. XVI, No. 64, October, 1952.]

Maheshwari (1942) points out that the antipodals may be situated in a narrow pouch.

The synergids are described as extremely long by Lyon (1898), while Weniger (1917) states that the egg protrudes beyond the synergids.

The present series of investigations was commenced mainly with a view to elucidate the embryological behaviour of the family.

Material and Methods

Euphorbia rothiana Spreng. is an erect glaucous annual with terete stems and oblanceolate, mucronulate leaves. The floral parts were fixed in Nawaschin's fluid. The dehydration and embedding in paraffin was done in the usual manner. Sections were cut at 12 microns in younger stages, but older stages were sectioned at 20 microns. Safranin-Gentian violet was used as the principal stain. Safranin-Delafield's haematoxylin was also used for older stages of the embryo.

Organogeny

The flowers are grouped together in a monoecious cyathium which is composed of a campanulate involucre, the margins bearing a number of pinkish crescent-shaped glands. The cyathium first appeared as a round protuberance of the peduncle and was surrounded by two bracts. Between these and the peduncle were developed the staminate flowers and involucral bracts. The carpels arose as papillate outgrowths of the conical receptacle and were inserted in a whorl (Figs. 1–4). The infolded margins of the carpels fused to form the trilocular ovary. The loculi were single-ovuled. The ovules were anatropous and pendulous with raphe towards the axis. The obturator concealed the micropyle by its elongated cells.

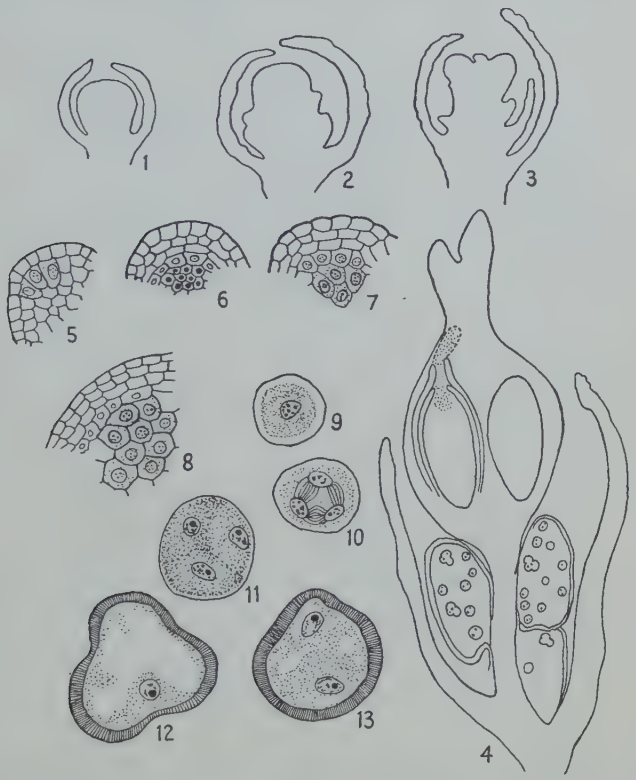
The development of inner and outer integuments was almost simultaneous.

Microsporangium and Male Gametophyte

A transverse section of the young anther lobe shows a row of one to three hypodermal archesporial cells (Fig. 5) which divide periclinally to form the primary parietal and sporogenous layers (Figs. 6–7). The wall of the anther is formed by further periclinal divisions of the primary parietal layer, while the spore mother cells originate from the division product of the sporogenous cells (Fig. 8). A tapetal layer could be distinguished in most preparations. The dividing microspore mother cells first became rounded and underwent the usual reduction divisions to form tetrads of microspores (Figs. 9–11). The microspores were delimited simultaneously.

The first division of the microspore cut off a lenticular generative cell and a large tube cell (Figs. 12–13). The cell-walls were not clearly defined. D'Amato (1947) states: 'Nelle altre 7 specie, si è osservato che i granuli pollinici più adulti sono già forniti del nucleo vegetativo e di quello generativo.' Probably in *E. rothiana* also the tube cell should be designated as a tube or vegetative nucleus as it does not possess a distinct wall of its own.

The mature pollen grain possessed only two nuclei. The exine was thickened by vertical striations. The protoplasm was vacuolated (Fig. 13). Pollen tubes and stages at the time of fertilization were not seen.



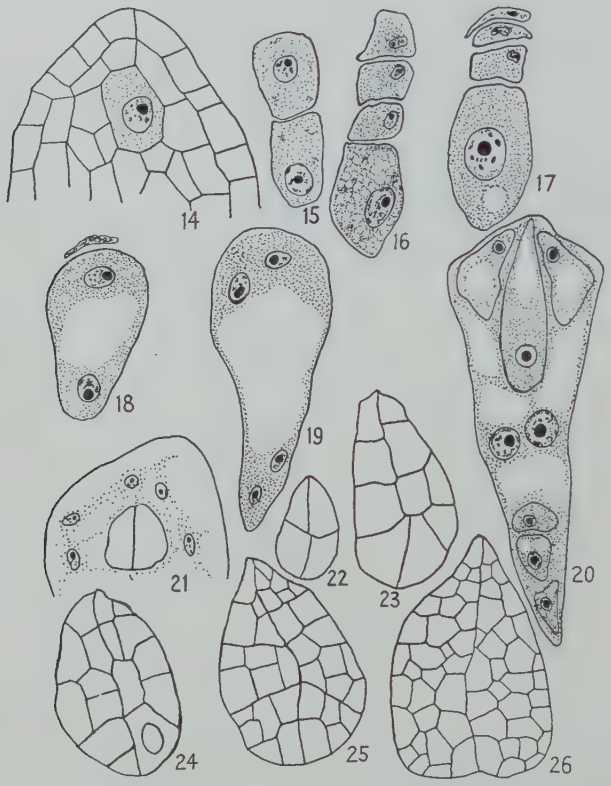
FIGS. 1–4. Stages in the development of the cyathium. ×26. FIGS. 5–8. Stages in the development of the microspore mother cells. ×320. FIGS. 9–11. Microspore mother cells in division. ×430. FIGS. 12–13. Microspores. ×430.

MEGASPORANGIUM AND FEMALE GAMETOPHYTE

Quite early in the development of the cyathium the ovule arose as a round swelling on the central papilla which was destined to form the carpel. This was followed by the inner integument. The outer integument started growth after the inner but soon surpassed it in extent. Coincident with the differentiation of the integuments was the appearance of a sub-epidermal archesporial cell. In all the preparations examined, the archesporial cell was solitary and could be distinguished from the cells of the nucellus by its large and deeply staining nucleus and dense cytoplasm (Fig. 14).

The archesporial cell functioned directly as the spore mother cell and gave rise to a linear tetrad of four megaspores, of which the large chalazal was functional. It contained dense granular cytoplasm and several small vacuoles (Fig. 17).

In subsequent stages of germination of the functional megaspore the disintegrating megaspores could be clearly seen. The nucleus of the functional megaspore divided to form the binucleate gametophyte. At this stage the several vacuoles fused to form a large central vacuole which served to shift the two nuclei to the two poles of the developing gametophyte (Fig. 18). The



FIGS. 14–17. Development of the megaspores. ×430. FIGS. 18–20. Development of the embryo-sac. ×430. FIGS. 21–26. Development of the embryo. ×320.

two nuclei next divided to form four and finally the eight-nucleate embryo-sac (Figs. 19, 20). Thus the development of the embryo-sac was of the normal eight-nucleate type.

The mature embryo-sac showed two well-defined synergids possessing posterior vacuoles. These occupy the micropylar part of the embryo-sac which appears to be pushed in between the integuments. The oosphere is pyriform and projects in the cavity of the sac beyond the synergids. It had a small but clear anterior vacuole. In all the embryo-sacs the polar nuclei remained distinct for a considerable period and no secondary nucleus (fusion product) could be seen. Due to a pronounced vacuolization of the sac, the polar nuclei occupied the centre of the sac (Fig. 20). Probably these nuclei unite immediately after the entry of the pollen tube (D'Amato, 1939). The antipodals were

placed in a row, one above the other, and the chalazal part of the sac was elongated in a pouch-like manner (Maheshwari, 1942). The antipodals were not ephemeral.

The process of fertilization could not be studied.

Endosperm and Embryo

The endosperm was free nuclear. In the post-fertilization stages the synergids appeared to have degenerated and the antipodals reduced in dimensions.

The endosperm initial divided much before the zygote showed any activity. When the embryo was only two-celled, the free nuclei of the endosperm had spread in a row at the periphery of the embryo-sac and surrounded the developing embryo (Fig. 21). The centre of the sac was, at this stage, occupied by a large vacuole.

The first division of the zygote was vertical and the second transverse (Figs. 21, 22). Further divisions transformed the embryo into a globular mass of cells (Figs. 23–26).

Up to this stage in the development of the embryo no suspensor could be distinguished.

Conclusions

The Euphorbiaceae exhibit a peculiar constancy in the development of the cyathium and in microsporogenesis, in view of the diverse types of female gametophytes. The development of the cyathium is initiated by the formation of an enclosed papilla (Weniger, 1917) and is followed by the origin of the staminate flowers and involucral bracts.

The microsporogenesis is normal in most species and the tetrahedral arrangement of microspores can be readily seen inside the spore mother cell (D'Amato, 1947). In *E. nutans* the adult microspore has been observed to be uninucleate, but in other species binucleate condition is frequent. Division of the generative cell into male nuclei has been reported by D'Amato (1947) to take place when the pollen tube reaches the base of the oosphere. In *E. rothiana* the mature microspore was binucleate.

The archesporium in the Euphorbiaceae appears to vary in extent. Schmidt (1907) describes more than one megaspore mother cell in *E. palustris*. In *E. dendroides* (D'Amato, 1947) the archesporial cells range from one to three. Modilewski (1910, 1911) observed pluricellular archesporium of five to seven cells—in *E. procera* and *E. palustris*. In *E. rothiana* there was a single archesporial cell which functioned directly as the megaspore mother cell. In one case Modilewski (1910) has described a row of three to four archesporial cells, each with four nuclei. Of these only one developed into the embryo-sac. Such a disintegration of archesporial cells is very rare.

In the normal types of embryo-sacs, gametophytic development is preceded by the formation of a linear tetrad of megaspores. In all monosporic Euphorbiaceae this condition is present.

Several authors have described the prior development of the inner integument (Lyon, 1898; Weniger, 1917; Maheshwari, 1942; D'Amato, 1947). However, the outer integument shows more rapid growth (Schweiger, 1905; Maheshwari, 1942). This condition was present in *E. rothiana*.

The mature embryo-sac of *E. rothiana* contained angular synergids similar to those in *E. preslii* (Weniger, 1917), but the polar nuclei were present very close to each other, unlike *E. preslii*.

The endosperm in the Euphorbiaceae has been repeatedly shown to be of the nuclear type. In *E. rothiana* the division of the endosperm initial was very rapid. A similar case has been described by Weniger (1917).

Souèges (1924, 1925) has given a detailed account of the development of embryo in *E. exigua* and *E. esula*; and Weniger (1917) has described embryo development in *E. preslii* and *E. splendens*. In *E. rothiana*, however, the zygote divided by a vertical wall in the first instance. Further work on the embryogeny of the Euphorbiaceae is necessary before any conclusions are reached on this point.

Summary

The cyathium in *Euphorbia rothiana* Spreng. arises as a papilla on the apex of the peduncle. The staminate flowers and involucre appear simultaneously, but carpels are formed last.

The microsporangium develops the pollen grains from a subepidermal archesporium of one to three cells. The exine of the mature binucleate pollen is thickly striated.

The stalked ovary is trilocular and contains uni-ovulate loculi. The ovules are bitegmic and crassinucellate. A single megaspore mother cell gives rise to a linear tetrad of four spores. The functional megaspore is vacuolated and forms a normal, monosporic, eight-nucleate embryo-sac. The antipodals are not ephemeral.

The endosperm is of the nuclear type. The embryo divides by a first vertical wall and is soon transformed into a globular mass of cells. The development of the endosperm is more vigorous than the embryo.

Acknowledgements

I am grateful to Professor P. Maheshwari, Delhi University, India, and Professor D'Amato, Istituto Botanico, Pisa, Italy, for several valuable suggestions. To Professor Shri Ranjan, Allahabad University, I am very thankful for kind encouragement and interest.

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An Inhibitor of Salt Absorption in the Root Tissues of Red Beet

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With six Figures in the Text

Abstract

The absorption of manganese ions by discs of beet-root tissue occurs in two phases. Evidence suggests that the first is physical in nature, the second physiological. The interval between the two phases becomes longer as the thickness of the discs is increased and reasons are given for attributing this to the presence of an inhibitor of the physiological absorption process.

Aqueous extracts of beet-root contain an inhibitor of ion absorption which also delays the germination of mustard and cress seeds. The extract does not affect the respiration rate of beet-root tissue.

Introduction

STORAGE tissues of roots and tubers have been used for the investigation of salt absorption and respiration problems by many research workers over a long period of years, doubtless because these tissues were a convenient source of large quantities of fairly uniform plant cells which showed no morphological evidence of being adapted for any special function. From the way in which the results of this sort of experimental work were used it is clear that in many cases the absence of morphological evidence of specialization has been taken to imply the lack of physiological specialization and studies on storage tissues have often been considered to be applicable to plant tissues in general. In the matter of salt absorption with which this paper is concerned the results of experiments with storage tissues have been thought to illuminate the process of salt absorption by the apparently unspecialized absorptive cells of actively growing roots. That there should be a great degree of similarity between these two tissues in the matter of salt absorption seems very doubtful to the authors. The high concentrations of organic food materials and salts normally present in the cells of beet-root and the role of a more or less static food reserve during a period of dormancy between the vegetative and reproductive phases of the life-cycle would lead one to expect

[Annals of Botany, N.S. Vol. XVI, No. 64, October, 1952.]

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a highly specialized physiology in storage tissues differing considerably from that of the short-lived absorbing cells of active roots. Differences in the physiology of the same tissue at different times of the year might also be expected to occur since during the course of development of a beet-root or carrot the root-cells change over from a function mainly concerned with the absorption and translocation of salts and water to that of food storage. After a period of inactivity lasting about 6 months renewed growth of the plant results in the movement of organic food materials and salts away from the root instead of into it and probably a resumption at least on a limited scale of salt and water absorption from the soil. It is also unlikely that storage tissues in different species of plant are closely similar. Tubers such as those of potato, in which the developing shoots soon form their own root systems, may well differ from beet-root, in which the storage roots remain the main channel of supply to the shoots. It was the aim of the work described below to examine the capacity of beet-root tissue in the dormant condition to absorb inorganic salts in the light of its special function in the life-history of the plant.

Methods

The beet-roots used were of Sutton's 'Crimson Globe' variety taken from store between the months of October and May. Discs 20 mm. diameter and 1 mm. thick were cut from the inner parts of the root, washed for 20 minutes in three changes of distilled water, and placed in hard glass jars with perforated stoppers containing the solution. Prior to cutting the discs the roots were cut into large cubes and permitted to become fully imbibed by soaking in distilled water overnight. In the earlier experiments the discs were loose in the solutions, but later they were threaded on silver wires spaced by small beads to permit more readily the access of liquid to the surfaces. The jars were shaken continuously in a bath thermostatically controlled at 21° C.

The solution used for measuring absorption was 0·001 molar manganese chloride, and except in a few instances 20 discs were placed in 200 c.c. of solution. The absorption of the manganese ion was estimated by analysing with the polarograph small samples taken from the solutions at fairly frequent intervals in the early part of each experiment and at daily intervals afterwards. In a few experiments absorption of the anion was also measured by the polarograph. The polarographic method of analysis is substantially the same as described by Stiles and Skelding (1940). At first, when the solutions were not changed during an experiment, it was suspected that the falling concentration of salt was having an important effect on the rate of absorption. The method was therefore modified by replacing the solutions completely after each estimation. At a still later stage of the work it was realized that this procedure might be removing salts and perhaps other substances from the tissue and so influencing the rate of absorption of manganese. To overcome this difficulty a further modification of procedure was adopted. After each estimation small calculated quantities of a concentrated solution of

manganese chloride and distilled water were added to bring the manganese ion concentration and the volume of the solution in the jars back to its initial value.

In this way experiments could be carried on for a week or more with the tissue still apparently healthy and continuing to absorb manganese. The solutions were free from bacterial contamination. The amount of manganese lost by the external solution to the tissue was expressed in micromols (10^{-6} M.) per 20 standard discs, i.e. per 6·28 c.c. of tissue.

Results

I. *The two phases in the course of absorption of ions*

Many workers have recorded that when plant cells are placed in solutions of salts there is at first a very rapid absorption of the cations. This first phase is complete in about 4 hours and is succeeded, either immediately or after a delay which may be as long as 70 hours in some cases, by a phase of slow absorption which continues at a steady rate until the supply of cations is nearly exhausted. This two-phase course of cation absorption has been reported by S. C. Brooks (1937), Steward and Harrison (1939), Stiles and Skelding (1940), Robertson (1941), and Stiles and Dent (1946). There is no rapid initial absorption of anions. A slow steady absorption may begin immediately or after a delay corresponding in duration to that separating the two phases of cation absorption. Typical curves of cation and anion absorption are shown in Fig. 1, which illustrates the results of an experiment with manganese chloride.

Steward and Harrison (1939) consider that these facts indicate that there are two different mechanisms of absorption; that which operates during the first phase of cation absorption is mainly physical in character and may be compared with adsorption, whereas the other in which both ions are absorbed simultaneously in approximately equal quantities is of quite a different nature and accounts for the second phase. Stiles and Skelding (1940) took a similar view of the process, but claimed that when tissue was placed in solutions of single neutral salts, basic exchange between the cells and the solution was partly responsible for the cation absorption. Stiles and Dent (1946) stated that the first phase was conditioned by a Donnan equilibrium, whereas the second phase was the result of the onset of increased metabolic activity which followed the cutting of relatively inactive tissue. They showed that the duration of the delay in the commencement of the second phase of absorption could be reduced by washing the tissues in running tap-water.

The present experiments have confirmed the duplex course of cation absorption and provided strong evidence that the first phase (subsequently referred to as phase I) is physical in nature and the second phase (phase II) physiological. The chief interest of the work has, however, centred on the causes of the delay which separates the two phases.

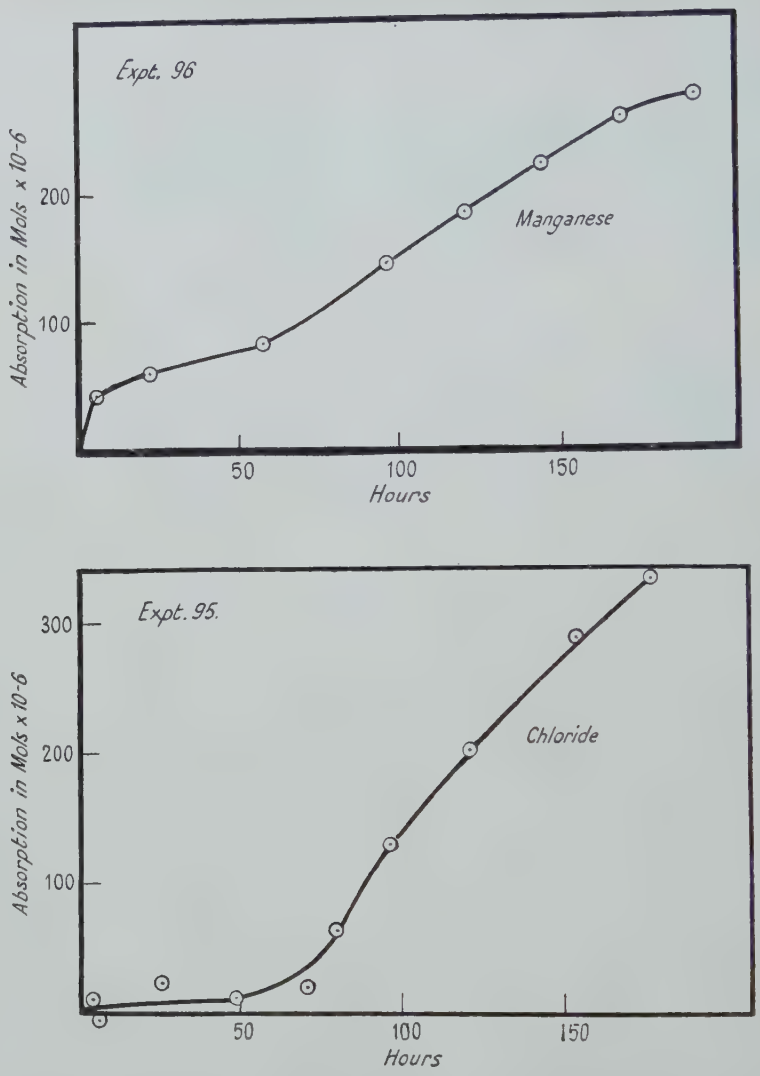

FIG. 1. The course of absorption by red beet tissue of the ions of manganese chloride from a solution maintained at a concentration of 0·001M.

II. *The physical nature of phase I absorption*

Many of the experiments which were designed to investigate other aspects of the salt absorption process have incidentally provided evidence of the physical character of phase I. The evidence may be stated briefly as follows.

1. *Rapidity of phase I absorption.* The speed at which, in phase I, equilibrium is reached in cases where there is a delay between the two phases suggests a physical process. Equilibrium is reached in 2–4 hours after placing the discs in the solution, giving an average of more than 2·5 μM. per hour

per c.c. of tissue compared with about 0·2 μM. per hour per c.c. during the second phase of absorption.

2. *Temperature coefficient.* In expts. 97 and 100 absorption was compared at 1° C. and 21° C. Phase I absorption showed a Q_{10} of about 1·1 and phase II a value between 1·9 and 2·3.

3. *Anaerobic conditions.* In expts. 99 and 103 phase I was quite unaffected by prolonged treatment with nitrogen whereas phase II was almost completely suppressed in the absence of oxygen.

4. *Cation exchange.* Spectrographic analysis of the liquid around the discs showed that calcium escaped from the cells in greater quantity into a solution of manganese chloride than into distilled water.

5. *Antagonism.* In expt. 91 calcium chloride was added to the solutions in various proportions relative to the manganese chloride. The amount of manganese absorbed in phase I was progressively depressed by increasing amounts of calcium ion until, when the calcium was ten times the concentration of the manganese, the latter was not absorbed at all. The absorption of manganese in phase II, although reduced by the addition of calcium, was never stopped completely. A calcium to manganese ratio of 5:1 produced the maximum depression of the absorption rate in phase II, although it was still 73 per cent. of that from a pure manganese chloride solution.

The evidence cited provides strong support for the view that the first phase of cation absorption is a physical process probably caused by base exchange between the solution and the cytoplasm, although full proof of this would require accurate measurements of the quantities of ions exchanged and the changes of reaction in the solution. By contrast, the second phase, in which cations and anions are absorbed simultaneously, which is strongly influenced by temperature and oxygen supply, which is relatively unaffected by other antagonizing ions and which continues at a steady rate for more than 10 days provided the supply of salt is maintained, would seem to be essentially a physiological process.

III. *Evidence for the existence of an inhibitor of salt absorption in beet-root.*

A series of experiments was designed to investigate the reason for the 50-hour delay in the commencement of the physiological phase of absorption. Such a delay was customarily found in our experiments when the discs were given only a very short washing period (about 1 hour) between cutting and placing in the solutions. Stiles and Dent (1946) found that by prolonging the washing period before placing the discs in the absorbing solutions the onset of the second phase could be accelerated, the absorption of both cations and anions being affected. The authors considered that 'the lag period occurs while the metabolic activity is rising to a level necessary for accumulation to take place'. Rees (1949), who investigated the effects of various preliminary treatments on the salt absorption of root tissue discs, found that aeration with moisture-saturated air had the same effect as washing in shortening the delay between the two phases. It seemed likely, if the delay was a measure

of the time required for certain changes to take place in the condition of the cells resulting from the access of oxygen to the tissue, that its duration would be influenced by the ratio of the surface area to the volume of the discs. Experiments 94, 95, and 144 were designed to test this supposition.

1. *Effect of relative surface area on the course of salt absorption.* Discs were cut with five different thicknesses, namely 0·25, 0·5, 0·75, 1, and 1·5 mm. The numbers of each type were adjusted to give the same bulk of tissue as twenty 1-mm. discs and these were placed in 200 c.c. of solution. The absorption of

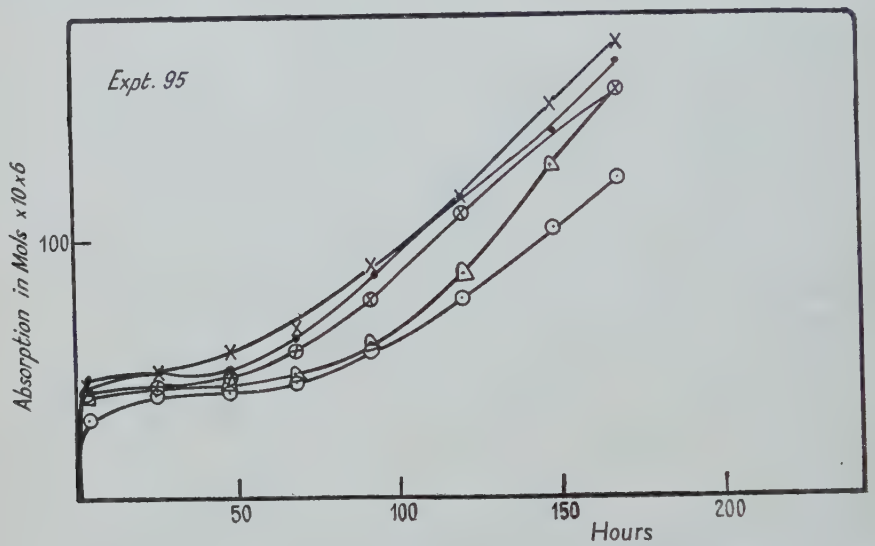

FIG. 2. The course of absorption by red beet tissue discs of varying thicknesses of manganese ions from a solution of manganese chloride maintained at a concentration of 0·001M. ● 0·25 mm. X 0·5 mm. ⊗ 0·75 mm. △ 1 mm. ⊙ 1·5 mm.

manganese ions is plotted against time in Fig. 2. Each point is the mean of two replicates. It will be seen that there is an immediate, rapid absorption of the cations in all cases. This is followed by a period of slow or zero absorption of which the length increases with increasing thickness of the discs. Finally, a slower steady rate of cation absorption is established and continues to the end of the experiment. There is no rapid initial absorption of the anion, but the establishment of the steady rate is preceded by a period of non-absorption comparable in length to that for the cation for the same thickness of disc. The quantity of manganese ion absorbed in phase I during the first 2 hours is very closely the same in all except the thickest discs which take about 6 hours to reach the same state of equilibrium. It was concluded that the manganese becomes equally distributed throughout the tissue at a fairly rapid rate. The amount of manganese absorbed in the first phase is therefore relatively unaffected by the distance of the cells from the surface and by any consequent differences in the oxygen supply which might exist,

a fact which supports the contention that a physical process is involved. The rate of movement of manganese ions into even the thickest discs (i.e. with smallest relative surface area) must be greater than 2·5 μM. per hour per c.c. of tissue during the early part of the process before the approaching equilibrium causes a slackening of the rate. In the 1·5-mm. thick discs the delay before the onset of the second phase of absorption is between 70 and 80 hours. The 0·25 mm. discs show scarcely any delay at all and intermediate thicknesses show delays of intermediate duration. In all cases a steady rate of manganese intake was finally attained which continued until the end of the experiment at 170 hours. This rate was very nearly the same for all except the 1·5-mm. thick discs, in which case it is slightly lower. The steady rate of manganese absorption does not seem to be related to the surface area or thickness of the discs but merely to the number of cells present. It seems, therefore, that a simple limitation of oxygen supply in the inner part of the discs could not be a factor restricting the rate of salt intake during the lag period especially as the lag may be as long as 70 hours. In the thicker discs there is virtually no absorption of manganese between the 20th and 60th hours of the experiment. If some external influence were concerned in the development of the power to absorb ions one would expect the outer layers of the thicker discs to behave in the same way as the thinner discs since they are similarly placed in relation to such influences. There would thus be some absorption taking place. The complete absence of absorption by the thicker discs for so long a time suggests that the inability of the cells to absorb ions when first cut from the root may be due to some internal inhibiting substance which is gradually removed by washing or by exposure to moist air. Such a substance would be removed more rapidly from thinner than from thicker discs, but, until removal was nearly complete, would continue to influence the surface cells through which it must pass in order to escape. Thus the thicker discs would have a longer period of non-absorption than the thinner discs, which was found by experiment to be the case.

The course of absorption of the chloride ion follows the same general course as the cation apart from the absence of the physical phase. The amount of cation and anion which have been absorbed by the time steady rates are established are very closely in the ratio of 1:2.

2. *Removal of the inhibitor by washing at low temperatures.* The hypothesis was put forward in the preceding section that the low level of metabolism of discs newly cut from storage roots was due to the presence of an inhibiting substance which could be removed by washing in running water. It was thought that the hypothesis might receive support if discs of tissue freshly taken from the root were washed under conditions unfavourable for the development of more active metabolism. The washing process should remove the inhibitor, and when the tissue was returned to suitable conditions salt absorption should begin at once or at any rate after a much reduced lag period. Two series of experiments were devised on this principle. In the first series low temperature was used to keep metabolic activity at a low

level during washing and in the second series exposure to an atmosphere of nitrogen.

In expt. 100 discs were placed in manganese chloride solution after a short preliminary wash in distilled water and the jars were then kept in a refrigerator which varied between 1° and 2° C. There were two different treatments applied to different batches of jars. In one set (*B*) the solutions were completely replaced after each sample had been analysed and in the other (*A*)

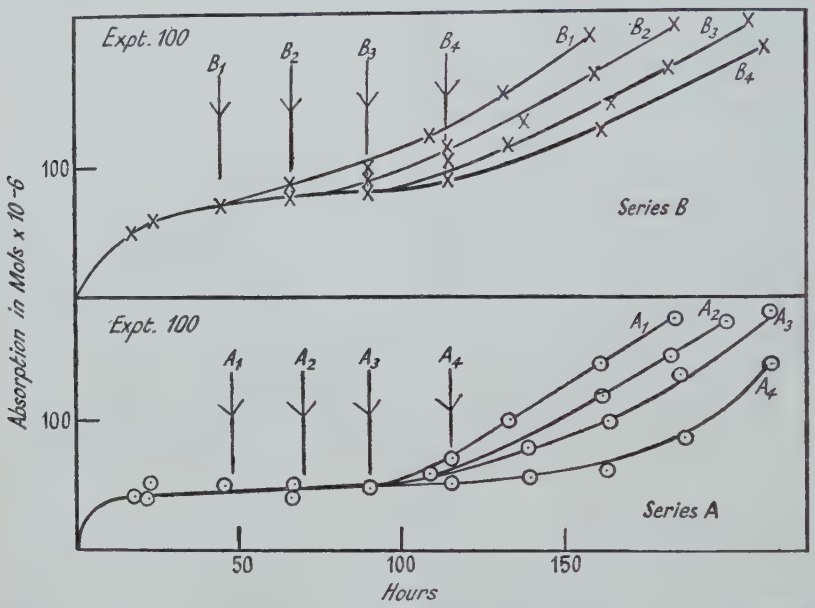

FIG. 3. The course of absorption by red beet tissue of manganese ions from solutions of manganese chloride maintained at a concentration of 0·001M. and at low and normal temperatures. Series A: Manganese chloride added to maintain concentration. Series B: Solutions changed. The times at which the solutions and tissues were changed from 1° C. to 21° C. are indicated by the arrows ⊥.

the manganese absorbed was replaced by adding small measured quantities of a strong solution. These two treatments were intended to expose the tissue to different degrees of washing. There were originally four replications of each treatment, but at intervals of 24 hours, beginning at 48 hours from the commencement of the experiment, jars were taken from the refrigerator to the shaker bath at 21° C. to test the ability to absorb salts. The manganese absorption is expressed graphically in Fig. 3.

It will be seen that the physical phase of absorption is scarcely different at 1° C. from that found in earlier experiments at 21° C. There was no 21° C. control in expt. 100, but using values from other experiments the Q_{10} over this range was about 1·1.

The subsequent absorption of manganese in treatment *A* is almost nil as long as the temperature was maintained at 1° C. Treatment *B*, however,

showed further absorption after the first sample and the graph of absorption against time gradually rose. This difference was attributed to the removal, when the solutions were changed, of ions (mainly calcium and potassium) which had been exchanged for manganese ions in the physical phase of absorption. The old equilibrium would be disturbed and more manganese ions would necessarily be absorbed.

The interesting comparison between treatments *A* and *B* is in the more rapid increase in manganese absorption in *B* after the jars were transferred

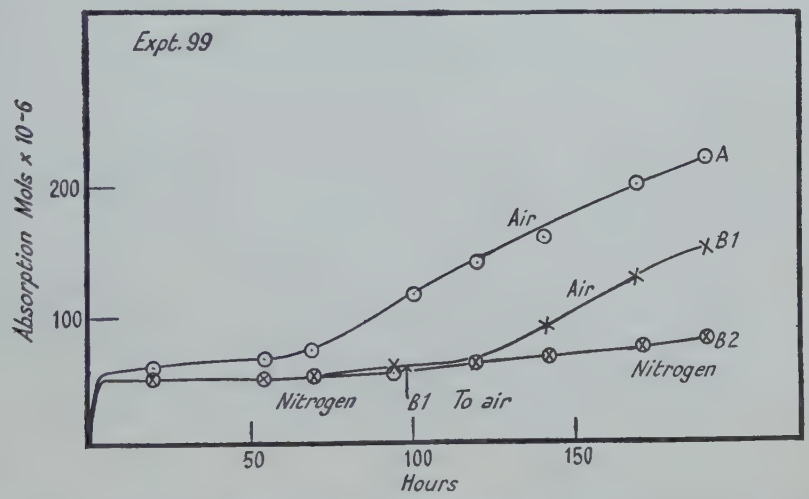

FIG. 4. The course of absorption by red beet tissue of manganese ions in air and nitrogen from solutions of manganese chloride maintained at a concentration of 0·001M.

to 21° C. In both *A* and *B* those jars which were transferred later showed a shorter lag period than the earlier ones and in all cases the lag in *B* was less than that for the comparable jar in *A*. In all cases the absorption rate finally attained is closely equal. This result is consistent with the hypothesis advanced.

3. *Removal of the inhibitor by washing at 21° C. in nitrogen.* In expt. 99 discs were placed in special containers through which nitrogen was bubbled, with controls through which air was passed. After 95 hours in nitrogen one set of replicates (B_1) were transferred to air and the remainder (B_2) were held in nitrogen until the end of the experiment. The manganese absorbed was replaced by adding quantities of a strong solution. The results are shown graphically in Fig. 4.

It will be seen that the physical absorption in nitrogen was identical with that of the air control. In nitrogen there was a slow absorption of manganese throughout the experiment, whilst in air there was the normal course of absorption with a lag lasting about 60 hours. The discs which were transferred to air after 95 hours in nitrogen were not injured by the treatment and began to absorb manganese after a lag period much shorter than for the air

control. The absorption curve is very similar to that of the *A* treatment in expt. 100 (Fig. 3) for a comparable washing period (90 hours in that instance). This result is also consistent with the hypothesis advanced.

4. *Removal of the inhibiting influence by oxidation.* In the foregoing section reference has been made to the removal of the inhibiting influence by washing the discs. This presumably may be attributed to the removal of a water-soluble inhibiting substance. In the light of this hypothesis work previously described by Rees (1949) also substantiates this opinion but shows that the inhibiting effect can be removed in other ways. He found that red beet pre-treated with air and nitrogen in distilled water showed an enhanced uptake of both ions as compared with the untreated tissue, the effect of air being greater than that of nitrogen and that of nitrogen greater than the untreated. When, however, the treatment was carried out on moistened tissue only the discs in air showed enhanced absorptive properties, whereas those in nitrogen were practically unaffected. The effect of gas treatment in water always greatly exceeded the action of the gas itself. The possibility of the dual effect of air and water was mentioned by this author. The earlier experiments of this paper substantiate the opinion that when freshly cut discs are placed in manganese chloride solution both dissolution and oxidation contribute to the removal of the inhibitor. The evidence described in this section for the existence of a salt absorption inhibitor in the root tissue of beet is all circumstantial. An attempt was therefore made to extract the active principle.

IV. *Extraction of the inhibitor*

De Kock and Hunter (1950) described the isolation of a yellow oil from water extracts of the seeds of *Beta* which acted as a germination inhibitor of mustard and other seeds. The experiments described above also indicate that the active principle in salt absorption inhibition is also water soluble. Consequently water extraction was attempted first.

1. *The inhibiting effect of water and chloroform extracts of beet-root.—Preliminary experiment 101.* Roots taken straight from store were cut into standard-sized discs, rinsed twice for 10 minutes in distilled water, and 200 discs placed in 200 c.c. of distilled water in a shaker at 21° C. After 24 hours the liquid was decanted and allowed to diffuse into a wide strip of filter-paper in the manner of a chromatogram. Complete evaporation of the 200 c.c. took about 8 hours in a cool dark room. The dry filter-paper strip was divided transversely into two equal parts and each part cut into small portions. The extracts were tested by adding the filter-paper to jars in which discs were absorbing manganese chloride. The discs were placed in the jars 48 hours before the addition of the filter-paper so that the lag period was then practically over and active absorption beginning. To one jar were added the torn-up pieces of filter-paper from the distal half of the strip, to a second jar the pieces from the proximal half, and to two others, similar quantities of unused filter-paper from the same sheet as was used for evaporating the extract, to act as controls.

In this experiment a chloroform extract of beet-root was also tested. The extract was made by grinding 90 g. of chopped beet-root with sand acidified with 60 c.c. of N/100 HCl in a mortar in the presence of 80 c.c. of chloroform. The paste was extracted with two further quantities of 30 c.c. of chloroform and the liquids separated in a separating funnel. The chloroform extract was then evaporated to dryness on filter-paper and half the filter-paper added to each of two jars of discs which were absorbing manganese. A sample was taken from the solution in each jar 15 minutes after adding the filter-paper to

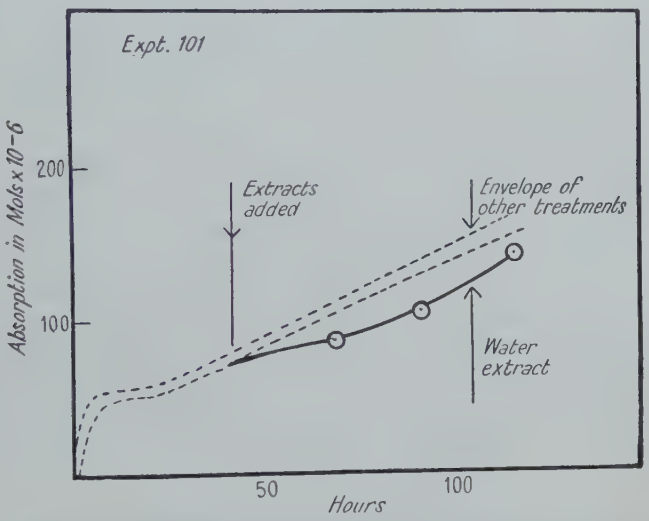

FIG. 5. The course of absorption by red beet tissue of manganese ions from solutions of manganese chloride maintained at a concentration of 0·001M., when water extracts of the tissue absorbed on blotting-paper were added.

guard against the possibilities that the filter-paper might adsorb the cation or that the extract may contain manganese. Two samples (at 24 and 48 hours) were taken before adding the extracts. They seem to indicate that the lag was only of about 24 hours' duration in this experiment, although the samples were too few to indicate the exact value. A shortening of the lag period had been noticed previously when the tissue had been stored for a long time (the experiment was performed at the end of May).

The results of expt. 101 are shown in Fig. 5. The dotted lines enclose the individual graphs of absorption for all the jars except that one to which was added the distal portion of the filter-paper containing the water extract. The early course of absorption shown in Fig. 5 is presumed, since the first sample was not taken until 4 hours from the start, but we know from other experiments that about 50μM. of manganese are absorbed in about 2–3 hours under exactly similar experimental conditions (see Fig. 1).

The results show that the more soluble part of the water extract of beet

tissue depresses the rate of manganese absorption but the less soluble part and the chloroform extract are not active in this respect. The sample taken 15 minutes after adding the extracts and filter-paper shows that there is no appreciable amount of manganese released from the extract or absorbed by the paper. In view of the possibility that the decreased rate of manganese absorption after adding the aqueous extract is due to the antagonism of the salts which water extracts of tissues always contain, the absence of an immediate loss of manganese from the tissue on adding the extract is important evidence that this is not the explanation. The decreased rate of cation absorption lasts no more than 48 hours, after which the rate is again equal to that in the control solutions. The salt absorption inhibitor is apparently fairly soluble in water but not in chloroform. With the objects of confirming the results of this preliminary experiment and the ultimate isolation of the active inhibitor, extractions were made on a larger scale and the extract was concentrated.

2. *Concentration of the aqueous extract.* Extracts were made from 1,000 discs by allowing them to stand for 24 hours in 1,000 c.c. of distilled water at 21° C. The liquid was then decanted and a second extract made with a similar volume of water. The extracts were reduced separately to a bulk of less than 100 c.c. by vacuum distillation at temperatures below 40° C. Each liquid was then made up to 100 c.c. and stored in a refrigerator. The concentrated extracts were always alkaline (pH between 8 and 9) and were therefore neutralized with 0·1 N hydrochloric acid before testing. The main test applied to the concentrated extract was its effect on the salt absorption of discs, but as the extract of beet fruits obtained by de Kock and Hunter inhibited seed germination, the effects of our extract on the seeds of mustard, cress, and oats were also tried. It was hoped that the latter might form the basis of a more convenient method of assaying the strength of an extract than the effect on salt absorption, which is too long for this purpose. Several other tests were later applied to the extracts to provide evidence as to the nature of the active principle. For the salt absorption test it was found that the extract concentrated ten times was not strong enough. A higher concentration was therefore prepared in which the extract from 5,000 discs was reduced to a bulk of 100 c.c. This is termed the 'super-extract'.

3. *Salt absorption test on the concentrated inhibitor.* The analysis of small quantities of the original concentrate (concentrated ten times) by Lindner's methods showed that it contained about 650 parts per million of potassium, 50 parts per million of magnesium, and 25 parts per million of calcium. Unpublished experiments by the authors on the absorption of salts from mixtures suggested that the salts present in the super-extract would be likely to cause an appreciable reduction in the rate of manganese absorption. It was therefore necessary to distinguish between the effects of the inhibitor and the salts unavoidably present in the extract. The method adopted in expt. 141 was the following.

Quantities of 10 c.c. of the super-extract were added to jars containing 20

discs in 200 c.c. of manganese chloride 48 hours from the beginning of the experiment at the time when the lag period is normally over. Four methods of treatment were applied in duplicate. In A_1 and A_2, the controls, only 10 c.c. of water were added. In B_1 and B_2 10 c.c. of the neutralized super-extract were added. In C_1 and C_2 10 c.c. of extract had been evaporated to dryness in silica basins over a low heat and redissolved in 10 c.c. of distilled water. In D_1 and D_2 10 c.c. of extract had been evaporated to dryness several times with a few c.c. of 20-volume hydrogen peroxide solution and then carefully

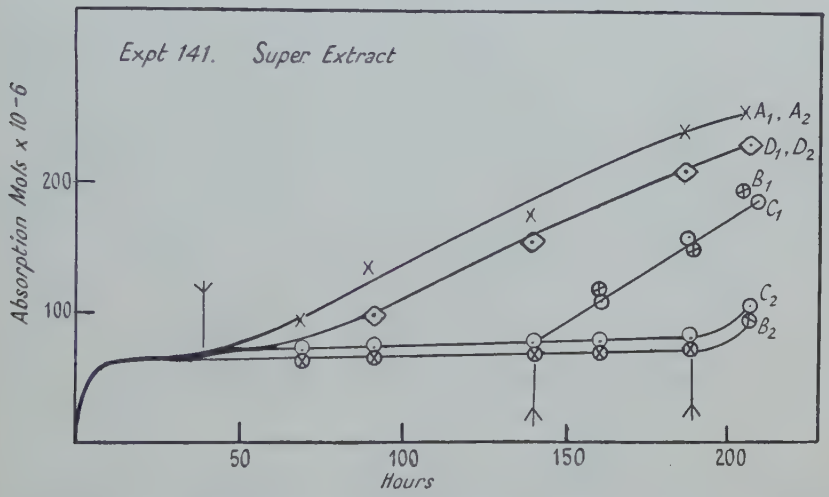

FIG. 6. The course of absorption by red beet tissue of manganese ions from solutions of manganese chloride maintained at a concentration of 0·001M. The time at which the respective extracts were added is indicated by the arrow ⊥ and the times at which the tissue was restored to pure manganese chloride are indicated by arrows ⊥. X no treatment; ◇ oxidized super-extract; ⊗ super-extract; ⊙ dried super-extract.

ignited. The residue was then redissolved in a little water, neutralized with 0·1 N hydrochloric acid, and made up to 10 c.c. In this way it was hoped that if the inhibitor was an organic substance it would be oxidatively destroyed but the salts would remain. The residues were almost completely soluble in water. The extract was added to D_1 and D_2 at the 48th hour. After each sampling for analysis the manganese which had been absorbed was replaced by adding an appropriate quantity of an 0·02 M. solution. The results are shown in Fig. 6. The course of absorption in the controls was completely normal. D_1 and D_2 showed the antagonistic effect of the salts in the extract. In B_1, B_2, C_1, and C_2 there was no salt absorption at all after the 48th hour.

After 142 hours B_1 and C_1 were placed in fresh solutions of manganese chloride. The rapid commencement of salt intake showed that the tissue was still capable of absorption. B_2 and C_2 were similarly transferred to new solutions after 190 hours and immediately began to absorb manganese. This

experiment shows conclusively that there is some salt-absorption inhibiting substance of an organic nature in the water extracts of beet-root tissue.

De Kock and Hunter (1950) state that the inhibitor which they extracted from the fruit of beet inhibited the normal development of seedlings of *Lepidium sativum* and other seeds, the effect being mainly on the radicles. Similar tests were therefore applied to the inhibiting extract from beet-root tissue.

4. *Germination tests.* The test was carried out in the manner described by Evenari (1949). Fifty seeds of mustard and cress were placed on a double thickness of Whatman No. 1 filter in a Petri dish. To this, 6 c.c. of the solution to be tested were added and the seeds incubated at 25° C. for about 2 days. Controls using distilled water instead of the extract were always used. Reports were made at frequent intervals. Ten cubic centimetres of normal extract (10:1) were neutralized approximately to pH 7 by adding 2 c.c. of 0·1 N hydrochloric acid. Six cubic centimetres of this solution was used and 6 c.c. of distilled water as a control. A third set of seeds of both species were treated with 6 c.c. of the neutralized extract which had been boiled for 5 minutes. The results of the experiment are shown in Table I.

TABLE I

Percentage Germination of Seeds after Various Times

Expt. 116.

	Time interval (hrs.)	Control.	Inhibitor.	Boiled Inhibitor.
Mustard	25	86	4	0
	48	90	6	0
	67	90	74	18
Cress	25	48	6	6
	48	68	8	8
	67	72	16	14

The criterion of a seed having germinated was the appearance of a radicle not less than 1 mm. long. The results show that the inhibitor causes a delay in the germination of both types of seed. The effect was not removed by boiling the extract and in the case of mustard seeds is inexplicably enhanced by boiling.

Further experiments of this sort which it is hoped to publish in a later paper have shown that the inhibitor influences only the earliest stages of seed germination, since, once the radicles have grown more than a few millimetres in length, there is no further effect.

V. *The inhibitor and respiration*

De Kock and Hunter reported that the germination inhibitor from beet seed depressed the respiration rate of root tissue. The inhibitor extracted

from beet-root tissue has not been found to affect either the oxygen consumption or the carbon-dioxide evolution of slices of beet-root tissue.

Discussion

In the foregoing account evidence is presented which strongly suggests the existence in dormant roots of red beet tissue of a growth and salt absorption inhibiting substance. The account represents the progress of the investigation up to the time of writing. For final proof that an inhibitor does exist in beet-root and in fact has the properties of salt absorption and growth inhibition it will be necessary to isolate the substance or substances responsible for the experimental facts, test them for purity, and if possible establish their chemical identity. This task will be undertaken next.

In view of the weight of evidence for an inhibiting substance it might be of value to speculate upon the role which it may play in the life-history of a root storage, biennial plant. The researches of Thornton (1945) have suggested that the accumulation of metabolic by-products which are not oxidized under the partly anaerobic conditions in the interior of large storage organs is responsible for the low level of metabolism during dormancy. This is possibly the mode of origin of the salt absorption inhibitor. The tissue of beet-root is formed from cambia which arise successively from the centre outward. As the tissue increases in bulk the inner layers will be subjected to increasingly anaerobic conditions. If such conditions lead to growth inhibitor formation, the inner cells including the inner cambia fall into a state of partial narcosis in which cell division and growth ceases and the ability to absorb salts is greatly reduced. Steward, Preston, and Ramamurti (1943) stated that the capacity of tissues to absorb salts is in proportion to their capacity for cell division and growth. Robertson and his fellow workers in a series of papers (1941–5) have shown that salt absorption is an energy-consuming process, so that it would be reasonable to anticipate a reduced capacity for salt absorption under conditions of narcosis. This is, in fact, what Robertson and Turner (1945) found when narcosis was induced by treatment with cyanides.

Evidence of this character would indicate that the primary effect of the inhibitor is to lower the general level of metabolism of the cell, which manifests itself in a reduction of growth such as was found in the germination test with mustard and cress seeds and in a reduced capacity for salt absorption as demonstrated in tests with discs which were already absorbing salts.

If this view is correct, it is to be expected that the inhibitor would also depress the respiration of the tissue, which the present authors have not been able to confirm. It is worth while considering, therefore, the possibility that an alternative explanation may be the correct one. Experiments not described in this account have shown that the effect of the concentrated inhibitor on seed germination was mainly in the very early stages of the process. Once the radicles had emerged more than a few millimetres the effect on the growth rate was slight. This suggests that the effect of the inhibitor may possibly

have been on the initial absorption of water by the tissues of the embryo. If the inhibitor reduces the permeability of the cytoplasm, then both water absorption and salt absorption are likely to be affected, but not necessarily respiration, which may be limited by internal factors rather than by the permeability of the membranes to oxygen and carbon dioxide. To decide between these two possible mechanisms of inhibition is a matter for further research.

In a plant such as beet-root with a biennial life-cycle the formation of an inhibitor may play an important role. By maintaining metabolism at a low level the organism is protected from premature development. If the inhibitor reduces the permeability of the cell membranes, the food reserves and accumulated supplies of inorganic salts may be protected from loss by leaching during the winter exposure. The gradual destruction of the inhibitor during the dormant period may be a kind of timing mechanism permitting development to occur after an appropriate lapse of time. In the course of the experiments recorded here there has been evidence of the disappearance of the inhibitor from beet-root about the end of May after a storage period of 8 months or so. The relationship of the inhibitor to the life-cycle is an aspect of the matter which is now receiving attention.

Summary

1. The course of absorption of manganese ions from a 0·001 molar solution of manganese chloride by discs of beet-root tissue was investigated.

2. The absorption of the manganese ion occurred in two phases separated by a period of about 50 hours in which there was practically no absorption.

3. Experiments indicated that the first phase, in which absorption was at first very rapid but soon ceased, was physical in nature, whereas the second phase of slower but sustained absorption was probably physiological.

4. Experiments with discs of different thicknesses indicated that manganese soon becomes evenly distributed through thick tissue in the first phase. The interval before the second phase of absorption began became longer with increasing thickness of the discs and reasons are given for concluding that the cause of the delay is the presence of an inhibiting substance in the tissue.

5. Evidence supporting this hypothesis was obtained when discs were washed under anaerobic conditions or at low temperatures. Although these conditions were unfavourable for developing a more active metabolism in cells, the interval which elapsed between returning the tissue discs to aerobic conditions or normal temperatures and the commencement of active salt absorption was reduced by the process of washing.

6. Aqueous extracts of beet-root contain a principle which inhibits for a time the absorption of ions by discs. The principle could not be extracted in chloroform.

7. Water extracts of beet-root tissue were concentrated fifty times by vacuum distillation at temperatures below 40° C. Such concentrates prevented completely the absorption of manganese ions for long periods of time without causing any apparent injury to the cells.

8. The concentrated extract containing the inhibitor also delayed the germination of mustard and cress seeds, the effect being mainly in the early stages of the process.

9. The concentrated extract did not affect the respiration rate of beet-root tissue.

10. The mechanism of inhibition and the role of the inhibitor in the biennial life-cycle of beet are discussed.

Acknowledgements

Some of the apparatus used in the course of this work was purchased with grants from the Research Committee of the University of Birmingham and the Government Grant Committee of the Royal Society, for whose assistance we are most grateful.

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Addenda Clavariacea

II. *Pterula* and *Pterulicium*

BY

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With twenty-five Figures in the Text

ABSTRACT

Revised descriptions are given of *Pterula Bresadoleana*, *P. capillaris*, *P. densissima*, *P. juruensis*, *P. multifida*, *P. plumosa*, *P. secundiramea*, *P. subulata*, and *P. taxiformis*. Twelve new species are described from tropical America.

A key is given to the American species of *Pterula* (28 spp., and 6 dubious spp.).

Reference to the types of *P. multifida* and *P. subulata* shows that the two species have been much confused and transposed.

The remarkable *Lachnocladium moniliforme* is transferred to *Pterula*. Some additional records of *Pterulicium* are given.

DRIED specimens of *Pterula* are hard, brown, and twisted, and so unlike the pale, delicate, living counterparts that herbarium-material may be very misleading. Such was my difficulty in studying the genus for my monograph, when I was acquainted only with living material from Malaya. Indeed, it is doubtful if any systematist of the genus has seen more than one or two species alive. I have since collected fourteen species in the living state in tropical America and *P. subulata* in England. This new material has enabled me to interpret the dried collections with some certainty and it has also shown the importance of the branching of the fruit-body. Accordingly, I have turned back to the herbarium-material, so far as it has been available, and publish here my results on the European species, *P. multifida* and *P. subulata*, the identity of which has been transposed, on the American species, and on such as have come to hand. I have been very greatly helped by the Lloyd Collections, most kindly loaned to me for study by Dr. John A. Stevenson, U.S. Department of Agriculture, Beltsville, Md.; by Friesian material, kindly loaned by Dr. Seth Lundell, Institute for Systematic Botany, Uppsala; and by material loaned by the Director of the Royal Botanic Gardens, Kew.

Excepting the north temperate *P. plumosa* and *P. subulata* and, possibly, their neo-tropical counterpart *P. Uleana*, none of the other twenty-five species that I describe from the New World have been found in the Old. Certainly the common Brazilian species are not the common Malayan species, and certainly there is much greater development of these fungi in the American than in the Asiatic tropics. In view of such strange forms, too, as those of *Allantula*

[Annals of Botany, N.S. Vol. XVI, No. 64, October, 1952.]

(Corner, 1952) and *Pterula moniliformis*, there is much yet to be discovered in the American tropics.

Branching. The fruit-bodies of *Pterula* may have apical or adventitious branching, rarely both (as in *P. Uleana*). Apical branching may be *multifid*, when the apex fans out and divides into several growing-points, each becoming a branch, or simply dichotomous. Multifid branching always occurs in one plane, though the planes of successive branchings may be more or less alternate, and thus the axil, or part of the stem subtending the branching, is more or less flabellate. Polychotomy, in which the apex divides into several branches in different planes, as in *Ramaria*, does not occur in *Pterula*. None of the American species, moreover, show the pseudo-monopodial state of the Malayan *P. verticillata*. Adventitious branching, on the other hand, is merely excrescence from the side of the main stem and does not involve the apex, so that the stem is truly monopodial: the excrescences grow out as monopodial branches and may branch again to a high degree, as in the very large fruit-bodies of *P. loretensis*. To make out the manner of branching it is desirable to have all stages in the development of the fruit-body.

Herbarium-material. If a dried fruit-body, or branch, is immersed in 3 per cent. potash, it swells, unravels, and regains its natural size and shape in about a minute. It can then be examined under the binocular microscope and the details of the branching can be made out. By such simple means most uninviting material can be made attractive and critical. Nevertheless, field-notes are needed for correct measures of the thickness of stem and branches, and for the colour, which may well be specific.

Key to the Pteruloid Fungi of the Americas

Gloeocystidia present: either with dichophyses or with papillate skeletal hyphal ends in the hymenium: with resupinate, corticioid hymenium . . . . . *Parapterulicium**

Without such features

Fruit-bodies decumbent, as small, intercalary swellings of the rhizomorphs: cystidia thin-walled, becoming immersed: on wood . . . . . *Allantula**

Fruit-bodies inverted, fasciculate, simple or branched: spores often angled in optical t.s., at least in dried specimens: on wood . . . . . *Deflexula**

Fruit-bodies erect: on the ground or on plant-remains . . *Pterula*

Key to the American Species of Pterula

With hymenial cystidia: small, simple or sparingly branched: tropical . . . . . Group 1

Without hymenial cystidia

Stem brown villose or brown setulose, from fascicles of hyphae or caulocystidia: tropical . . . . . Group 2

Without caulocystidia or such fascicles of hyphae

Simple . . . . . Group 3

Branched . . . . . Group 4

* Ann. Bot., New Ser., Vol. XVI, No. 62, 1952, p. 269.

GROUP 1

(with hymenial cystidia: small, simple or sparingly branched)

Cystidia thick-walled, encrusted at the apex: extreme base of the stem sclerotioid: sp. 10–12 × 5 μ: dead leaves, Brazil . . *P. cystidiata*
Cystidia thin-walled, not encrusted
Sp. 16–19 × 7·5–9 μ: stem not sclerotioid: wood, Brazil . . *P. navicula*
Sp. 8–11·5 × 4 μ: stem surface sclerotioid: twigs, leaves, Panama *P. epiphylloides*

GROUP 2

(no cystidia: stem brown villous or brown setulose)

Stem villous, the hyphae more or less fasciculate, the surface not sclerotioid: no caulocystidia: –2 cm. high, monopodial, the branches adventitious: sp. 5–7 × 3–4 μ: wood
Stem 0·7–1·5 mm. thick, strigoso-villous: rather stout: brownish with flesh-coloured branches: Brazil . . . . . *P. juruensis*
Stem 0·1–0·5 mm. thick, strigoso-hispid at the base or merely fibrilloso-subtomentose: slender: white, fuscous brown below: S. America . . . . . *P. taxiformis*
Stem setulose with caulocystidia, the surface sclerotioid
Sp. 7–9 μ long: sclerotioid surface 2 or more hyphae thick: –4 cm. high, branched
Sp. 3·5 μ wide: monopodial: dead leaves, Brazil . . *P. fluminensis*
Sp. 4–6 μ wide: multifid-dichotomous: wood, Ecuador . *P. velutipes*
Sp. longer: sclerotial surface 1 (–2) hypha thick: dead leaves, Brazil
Sp. 11–14 × 4–5 μ: –5 cm. × 0·5–1 mm., mostly simple . *P. brunneosetosa*
Sp. 13–20 × 4 μ: –2 cm. × 0·1–0·3 mm., with a few adventitious branches . . . . . *P. longispora*

GROUP 3

(without caulocystidia: small, simple)

Stem-surface sclerotioid: dead leaves
Sp. 10–15 × 4–5 μ, or 9–11 × 5 μ: temperate, tropical . . *P. tenuissima*
Sp. 6–8 × 2·5–3 μ: Brazil, Argentina . . . . . *P. tenerrima*
Stem not sclerotioid
–1·5 mm. high: twigs, branches
Sp. 18–20 × 10–12 μ: Ecuador (? *Deflexula*) . . . *P. macrospora*
Sp. 7–10 × 5–6 μ: Guadeloupe . . . . . *P. nana*
10–20 mm. high
Sp. 8–10 × 4 μ: gen. hyphae clamped: dead leaves, Brazil . *P. Gordius*
Sp. ?: without clamps: bark, Cuba . . . . . *P. subulaeformis*

GROUP 4

(without caulocystidia: medium to large, branched)

With swollen articulate joints in the lower part of the fruit-body: branches rather stout, dilated at the axils: sp. 6–7 × 3·5 μ: on the ground, Brazil . . . . . *P. moniliformis*
Without swollen joints: normally pteruloid with slender branches
Sp. more than 7 μ long: branches mainly or wholly adventitious: tropical

Sp. 7–9×4–5 μ: –4·5 cm. high, monopodial, branches adventitious, mostly simple: twigs, Brazil . . . *P. stipata*

(Branches copious, ? not monopodial: Peru . . *P. capillaris*)

Sp. 9–11×5–6 μ

Monopodial with simple secund branches: –3 cm. high: dead wood, Brazil: (? *Deflexula*) . . . . *P. secundiramea*

Multifid-dichotomous, not secund: –5 cm. high: dead palm-leaves, Brazil . . . . . *P. palmicola*

Sp. mostly less than 7 μ long: generally multifid below, dichotomous above

–12 cm. high, very bushy, with many secund adventitious branchlets: stem 3–5 mm. thick: sp. 4–5×2·3–3 μ: wood (? humicolous), Peru . . . . . *P. loretensis*

–2·5 cm. high, very slender, monopodial with simple adventitious branches: stem 0·1–0·3 mm. thick, appressedly fibrillose to substrigose, becoming fuscous: sp. 4·5–6·5×3–3·7 μ: wood, bark, tropical . . . . . *P. taxiformis* var. *gracilis*

Not so: 3–6 cm. high: sp. 4·5–7 (–8) μ

Sp. 3·5–4·7 μ wide: branches anastomosing, fastigiate: stem immersed in the ground, North America

–6 cm. high, pale brown tinged flesh-colour . . . *P. subulata*

–4 cm. high, pulvinate-congested . . . . *P. densissima*

Sp. 2·5–3·7 μ wide: branches not anastomosing

North America: multifid-dichotomous: stem 1–2 mm. thick: sp. 6–8 μ long: on the ground . . . . *P. plumosa*

South America

Branches compressed, strongly flattened at the multifid axils, tips elongate: on the ground, Brazil . . *P. complanata*

Branches more or less terete

Branches mainly dichotomous, divaricate strongly, hymenium unilateral: fuscous drab or tinged flesh-colour: stem prominent, –25×1–2 mm.: in humus, Brazil . . . . . *P. plumosoides*

Finally with adventitious branchlets, bushy, more or less fastigiate: pale ochraceous flesh-colour: stem –15×0·2–1·5 mm., not conspicuous: wood, bark (? in humus), Brazil, Argentina . . . . *P. Uleana*

Dubious Species

–9 cm. high, fastigiately branched: wood, Mexico . . . *P. angustata*

–2 cm. high, cinereous, moss-like: stems fuscous black: mossy trunks, Paraguay . . . . . *P. adustipes*

1 cm. high, stem with a whorl of branches at the top: branches furcate or verticillately branched: sticks, leaves, Brazil . . *P. pusilla*

3–8 mm. high, mostly simple

On herbaceous stems, Brazil . . . . . *P. falcatula*

On plant-remains: simple or 1–3 branches: Guadeloupe . *P. laxa*

On wood: flesh-colour: S. America . . . . . *P. incarnata*

P. aciculaeformis Lloyd

Type: Lloyd Cat. No. 32712 (Mt. Maquiling, Luzon, P.I., on dead wood, 1917).

This is a horse-hair fungus. The specimen consists of three light fuscous drab rhizomorphs, –7·5 cm.×0·3 mm., glabrous or slightly appressedly fibrillose. The outer layer consists of 2–3 μ wide hyphae with thickened agglutinated walls turning fuscous black in dilute potash. The inner hyphae, 2–8 μ wide, are colourless, clamped, monomitic, though many have strongly thickened walls (glassy opalescent in dilute potash); there are no caulocystida.

It is probably the mycelium of a *Marasmius* or *Crinipellis*.

P. Bresadoleana P. Henn.

Duplicates of the type-collection in the British Museum (Huhlmann 2517, Ruwenzori forest, *c.* 950 m. alt., July 22, 1891) and in Saccardo's herbarium (Central Africa, Wakondjo, leg. Huhlmann) show that this species is a true *Pterula* but of uncertain affinity. The small, nearly globose, spores are very abundant and are unlike the usual pip-shaped spore of the genus. The dimitic construction is evident, but typically imperfect in that some of the skeletal hyphae continue as generative hyphae, though the majority are long, more or less thick-walled, and unbranched. The following is a revised description.

–6 cm. high, caespitose, 3–5 times dichotomous, or the lower branchings multifid, glabrous, drying contorted, very horny and brownish to brownish ochraceous: stem –17×1 mm.: branches 0·5–0·7 mm. wide below, tapering but not filiform, the internodes rather long.

Spores 4–5×3·5–4·5 μ, white, subglobose, smooth, the wall slightly thickened, very abundant, the apiculus 0·5 μ long.

Basidia mostly decayed, but a few sterile basidia with thickened walls, measuring –45×7–8 μ, with 4 sterigmata, immersed in the thickened hymenium (–80 μ): cystidia none.

Hyphae dimitic: skeletal hyphae 4–7 μ wide, the walls 0·3–1·5 μ thick, possibly submucilaginous, occasionally secondarily septate without clamps: generative hyphae 2·5–5 μ wide, thin-walled, the clamps often large, mostly 2–3·5 μ wide near the subhymenium.

P. brunneola Corner

Lloyd Cat. No. 29258 (Belgian Congo, leg. H. Vanderyst, Nov. 1922), though without colour-note, clearly belongs here. It has the large fruit-bodies with rather stout branches, and spores 5–6×3 μ. It is the first record of the species outside Malaya.

P. brunneosetosa sp. nov.

Fig. 1.

Simple or sparsely branched, scattered or solitary, cylindric with acute tip,

rather stout, white or pale cream, becoming pallid tan, dingy alutaceous, or isabelline, with pale fawn brown, to fuscous fawn, brown-hairy stem; epiphyllous. –5 cm. high: fertile part 0·5–1 mm. thick: stem –12×0·5–1 mm., distinct, erumpent: branches adventitious, one or two, or clustered near the apex and more numerous, the tip even penicillate, generally unbranched: tough, without smell.

Fig. 1. *Pterula brunneosetosa*, nat. size: spores, ×1,000 (Corner 776).

On dead leaves in the forest: Tropical America (Brazil, Est. do Rio Niteroi, Corner 776, Aug. 31, 1947, type; Corner s.n., Sept. 21, 1947; Rio de Janeiro, Corcovado, *c.* 500 m. alt., Dec. 5, 1948, Corner 777: Bolivia, Cobija, Corner 778 and s.n., Feb. 1, 1948).

Spores 11–14×4–5 μ, white, smooth, rather fusiform-subamygdaliform, the walls slightly thickened, aguttate.

Basidia 30–38×8–10 μ, clavate to subventricose: sterigmata 4, 7–8 μ long.

Hymenium not thickening: cystidia absent.

Hyphae dimitic: skeletal hyphae 4–6 μ wide, the walls 1–2 μ thick, aseptate, colourless, unlimited: generative hyphae clamped.

Caulocystidia –150×8–20 μ, conical, acute, aseptate, smooth, with pale brown walls 0·5–1·5 μ thick, often sub-bulbous or flattened triangular at the base, abundant.

Stem covered with a thin sclerotioid epithelium (one hypha thick) of pavement-like cells, 5–7 μ wide, with brown, wavy, thickened walls, immersing the bases of the caulocystidia, but without hypodermal layer.

This striking species appears to be common, yet I have been unable to detect it among those already described from South America. The Brazilian specimens were all growing on the dead leaves of the same species of tree, but the Bolivian collections were each from a different species. The structure of the stem-surface is that which I have illustrated for *P. typhuloides* (Monogr., Figs. 232, 234).

P. capillaris (Lév.) Sacc.

This was described from Java and Peru. There is a fragment of the Peruvian type (leg. Dombard, Peruvia) in Bresadola's herbarium, U.S. Dept. of Agriculture Mycological Collection. The packet contains what appears to be a pencil sketch and a few fragments of branches. Examination of one of these fragments gave the following details:

Spores 7·5–9×3·7–4·5 μ, white, smooth, thin-walled, subcylindric to pip-shaped, the apiculus small, *c.* 0·5 μ long: (these spores typical of *Pterula*, not numerous but sufficient, in the absence of any other spores, to be sure of their identity).

Hymenium? thickening: cystidia none.

Skeletal hyphae 3–6 μ wide, rather thin-walled (? immature): generative hyphae 2–4 μ wide, clamped.

The species keys out near to *P. stipata*, with different branching (that of *P. capillaris* being apparently similar to *P. Uleana*), and to *P. palmicola* (larger spores). Lloyd said that he was unable to find the Javanese type of *P. capillaris*, in which case it would seem that the species must be defined on the Peruvian, though there is no microscopic evidence of their identity.

The Philippine specimens, on which Lloyd seems to have based his idea of *P. capillaris*, are *Pterulicium xylogenum* (B. et Br.) Corner, namely:

Lloyd Cat. No. 32739 (on dead wood, Los Baños, Laguna), with spores 10–12·5 × 5–6·5 μ, the skeletal hyphae 3–5 μ wide with walls –1·5 μ thick: fruit-bodies slender, multifid, then with adventitious branches.

Lloyd Cat. No. 32737 (on dead sugar-cane, Tela, Honduras), determined by Lloyd as *P. capillaris*, is also *Pterulicium xylogenum*, with spores 10–11·5 × 5–6·5 μ.

P. complanata sp. nov.

Fig. 2.

–4 cm. high, *caespitose*, *with flattened branching* (? in one plane), drying fuscous brown and horny: stem 2–8 × 1·5–2·5 mm., smooth or slightly fibrillar, *branching flabellately into several main branches* 1–2 mm. wide: *branches compressed, flattened and dilated* 2–5 mm. wide *at the multifid axils*, the upper branches apparently ligulate, *with acute, tapering, elongate and sparsely dichotomous branchlets*.

On clay banks: Brazil (near the city of Maranhão, leg. Gardner 1841, s.n.; in herb. Berk., Kew, ut '*Pterula plumosa* Fr. var. *acutissima* Berk.' ined).

Spores 6–7 × 3–3·5 μ, white, copious, elongate pip-shaped to subcylindric, smooth, the wall slightly thickened, the apiculus small.

Hymenium? thickening, but much decayed: cystidia none.

Skeletal hyphae 2·5–5 μ wide, the walls –1·5 μ thick: generative hyphae –5 μ wide, copious, clamped.

This species comes in the *P. multifida–P. plumosa* complex, with identical spores, but the strongly flattened branching of the fruit-body is characteristic. The type-collection consists of some 24 fruit-bodies, having a ragged appearance because of the elongate branchlets.

P. cystidiata sp. nov.

Fig. 3.

–15 mm. high, *simple*, rarely with 2–3 short adventitious branches at the apex, *filiform, white*, cream with age, 0·2–0·4 mm. thick, *with a very short, pale fuscous brown stem* and inserted base.

On dead leaves in the forest: Brazil (Est. do Rio, Niteroi, Saco de São Francisco, Mar. 7, 1948, Corner 768, *type*).

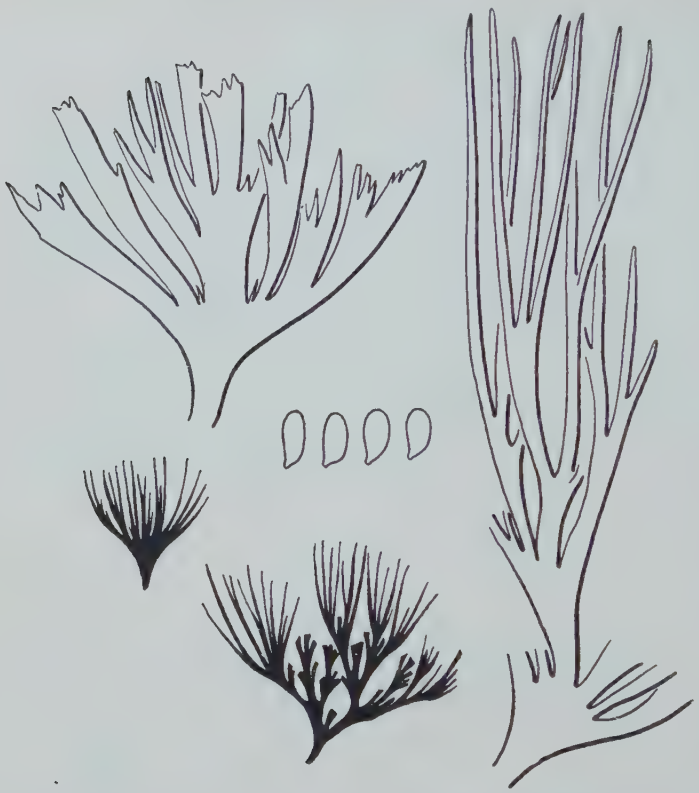

FIG. 2. *Pterula complanata*, fruit-bodies, nat. size: branches, ×3: spores, ×1,000 (type).

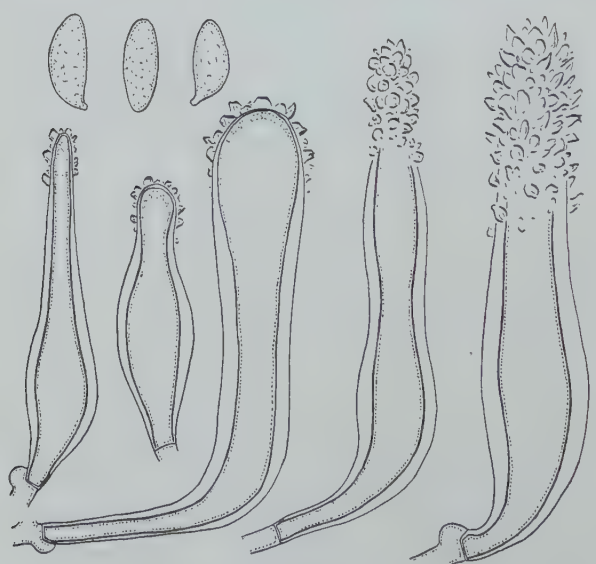

FIG. 3. *Pterula cystidiata*, cystidia and spores, ×1,000 (type).

Spores 10–12·5 × 4·5–5·2 μ, white, smooth, elongate-ellipsoid or subcylindric, thin-walled, aguttate, apiculus 1–1·5 μ long.

Basidia 30–37 × 9–10 μ: sterigmata 4, 6 μ long.

Hymenium not thickening, covering the whole of the fruit-body except the extreme apex and base for a length of 50–150 μ.

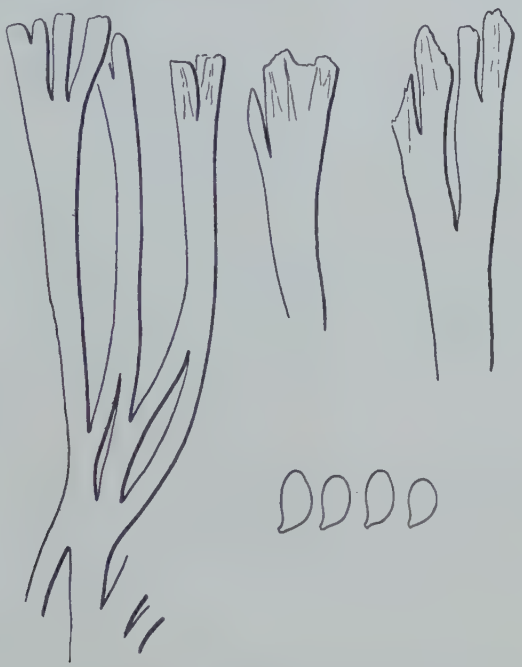

FIG. 4. *Pterula densissima*, branch-tips, × 20: spores, × 1,000 (type).

Cystidia 25–70 × 9–15 μ, the walls 1–3 μ thick, projecting –30 μ, subcylindric to subventricose or clavate, the apex thin-walled and rather *heavily encrusted* with crystalline matter for a length of 10–18 μ, rarely wholly encrusted, sometimes with a thick-walled subhymenial stalk –30 μ long, abundant.

Stem, at the extreme base, for a length of 20–60 μ, with brownish sclerotioid surface and with caulocystidia as reduced, but encrusted, hymenial cystidia.

Hyphae dimitic: skeletal hyphae 2·5–5 μ wide, the walls –1·5 μ thick, some unlimited, others as very elongate segments of generative hyphae, sometimes branched at the apex: generative hyphae 2–4·5 μ wide, clamped, walls thin or slightly thickened.

Compare *P. tenuissima* without cystidia.

P. densissima B. et C.

Grev. 2, 1873, 16: Lloyd, Myc. Writ. 5, 1919, 865, f. 1469.

Fig. 4.

–4 cm. high, densely *congested, pulvinate*, obconic, drying fuscous brown and horny: stem?, evidently immersed, but broken off the specimen: *branches* 0·5–1 mm. wide below, 0·5 mm. wide above, *multifid*, compacted, *much anastomosing*.

On the ground: U.S.A. (New England, leg. Sprague: type in herb. Berkeley n. 5324, at Kew).

Spores 5·5–7×3·5–4·3 μ, white, smooth, rather pip-shaped, with prominent apiculus, thin-walled.

Basidia 25–30×5–7 μ: sterigmata 4, 3·5 μ long.

Cystidia none.

Skeletal hyphae –6 μ wide, the walls slightly thickened: generative hyphae clamped.

I have examined the type of this species, which is known from the one collection. Macroscopically it suggests a densely branched state of *P. plumosa*, but the anastomosing branches and the wide spores show that it is nearer to *P. subulata*. The fruit-body, which is illustrated by Lloyd, seems to have proliferated a second set of branches over the surface of the original.

P. epiphylloides sp. nov.

–2 cm. high, 0·1–0·2 mm. wide, *simple*, filiform with acerose apex, *white*, *the sterile stem* (–8 mm. long) *becoming light fuscous brown* and darker at the inserted base.

On dead leaves and twigs: Panama (Barro Colorado Isl., July 31, 1935, G. W. Martin 3146, *type*: Fort Sherman area, Aug. 12, 1935, G. W. Martin, 6194).

Spores 8–11·5×3·5–4·5 μ, white, smooth, subcylindric, the apex subacute, the wall firm, the apiculus small.

Basidia?: hymenium apparently not thickening.

Cystidia –30×7–10 μ, clavate to subcylindric, thin-walled, hyaline, abundant, projecting as sterile basidia.

Caulocystidia –50×7–15 μ, varying short and clavate to ventricose with short and blunt or long, tapering and subacute apex, the walls brown, smooth, and slightly thickened, abundant.

Stem-surface sclerotioid, 1–2 cells thick, the superficial hyphae with brown, slightly thickened, gyrose walls.

Hyphae dimitic: skeletal hyphae 2·5–5 μ wide, –8 μ in places, the walls 0·5–1·5 μ thick: generative hyphae clamped, often branched from the clamp.

This species, like *P. epiphylla* of Malaya, appears a small simple ally of *P. debilis* of Europe, but with better developed caulocystidia. From *P. epiphylla* it differs in the sclerotioid surface of the stem and the less well-marked cystidia. On the other hand, it may be nearer the acystidiate alliance of *P. tenuissima* (no caulocystidia) and *P. typhuloides* (narrower caulocystidia, rarely acerose).

FIG. 5. *Pterula fluminensis*, fruit-bodies, ×2: spores and caulocystidia, ×1,000 (type).

P. fluminnesis sp. nov.

Fig. 5.

–3 cm. high, *monopodial, branches few to rather numerous, white: stem* –16× 0·2–0·4 mm., slender, *fuscous fawn, minutely brownish hairy*: branches –12× 0·1–0·2 mm., simple or with 1–3 adventitious branchlets: hymenium unilateral: drying horny, tough.

On dead leaves in the forest: Brazil (Corner 771, type, Aug. 31, 1947, and Corner s.n. Mar. 7, 1948, Est. do Rio, Niteroi, Saco de São Francisco: Rick 180 pr. p., Rio Grande do Sul).

Spores 7–8·5×3–3·7 μ, white, smooth, narrowly ellipsoid, thin-walled, aguttate, the small apiculus *c.* 0·5 μ long.

Basidia 20–25×7 μ: sterigmata 4.

Hymenium not thickening, cystidia absent.

Caulocystidia –120×7–14 μ at the base, conical, acute or subacute, the walls light brown and 0·5–1 μ thick, aseptate, the base often subbulbous and immersed in the sclerotioid surface of the stem.

Sclerotioid surface of the stem –15 μ thick, 2–4 hyphae thick.

Fig. 6. *Pterula incisa*, fragment of the type, ×5.

Hyphae dimitic: skeletal hyphae 2·5–4·5 μ wide, the walls –1·5 μ thick, unlimited or as intercalary segments of the clamped generative hyphae.

This is near to *P. brunneosetosa*, with stouter fruit-bodies, and *P. longispora*. Rick n. 180 consists of two different collections. One part, in the National Museum, Rio, and in Saccardo's herbarium, is *P. Uleana* P. Henn. The other part is the slender, epiphyllous *P. fluminensis*, also in Saccardo's herbarium, and it is to this that Bresadola must have referred when he doubted the identity of Rick 180 with Rick 24, which he made the type of *P. pusilla* (Ann. Myc. 18, 1920, 50). I have not been able to find Rick 24, and I am doubtful if *P. pusilla* is pteruloid.

P. incisa Lloyd

Fig. 6.

Type: Lloyd Cat. No. 32747 (Mt. Maquiling, Luzon, P.I., on wood, Oct. 4, 1920): 1 cm. high, penicillate-multifid at the tip, with very slender branches 0·05–0·2 mm. wide, sterile, without hymenium: skeletal hyphae 2·5–5 μ wide, well-marked: generative hyphae clamped.

This may be young *Pterulicium xylogenum*, which is common in the Philippines.

P. juruensis (P. Henn.) Corner

Figs. 7, 8.

–20 mm. high, scattered or gregarious, *bushy, monopodial with spreading, adventitious branches, pale to dark brown, or maroon brown below, the branches brownish flesh-colour to pale dull flesh-colour with white tips*, the hymenium whitish pruinose: *main axis* 0·7–1·5 mm. wide below, with a stem-like part 2–10 mm. long, *brown villous-hispid, then villous-tomentose* from more or less upwardly directed divergent fascicles of hyphae, the axis occasionally di- or tri-chotomous: *branches* 0·1–0·2 mm. wide below, tapering to fine, blunt, or filiform tips 10–50 μ wide, *simple or with a few adventitious branchlets*: tough.

On rotten wood and bark in the forest: Brazil (Amazonas, Rio Jurua, type: Est. do Rio, Niteroi, Aug. 31, 1947, Corner 782; Rio de Janeiro,

Corcovado, *c.* 500 m. alt., Nov. 20, 1948, Corner 772, and Nov. 25, 1948, Corner 752).

Spores 5–7×3·5–4·5 μ, white, smooth (but appearing finely rough after drying), broadly ellipsoid to pip-shaped, *1-guttate*, the wall slightly thickened, the prominent apiculus –1 μ long.

Basidia 18–28×7–9 μ, clavate with narrow base 2–2·5 μ wide: sterigmata 4, 5–6 μ long.

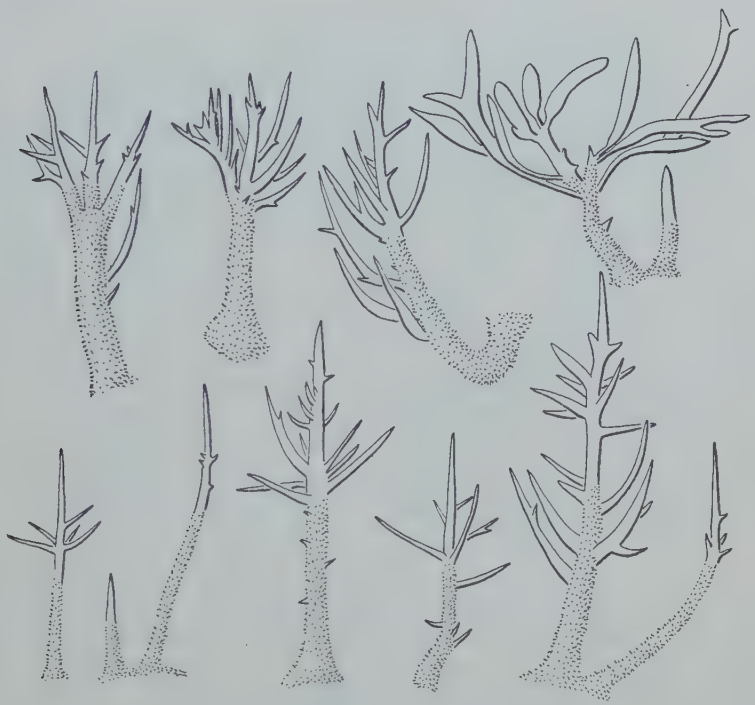

FIG. 7. *Pterula juruensis*, young fruit-bodies, ×5 (Corner 772).

Hymenium thickening to 60 μ: subhymenium slight: cystidia absent.

Hyphae dimitic: skeletal hyphae 3–5·5 μ wide, unlimited, the pale brown walls 0·5–1 μ thick, the aseptate lumen rather wide, rather few and ill-defined in the flesh of the stem, abundant in the branches: generative hyphae 2–5 μ wide, thin-walled, clamped, often branched from the clamp, very abundant in the stem and there often with slightly thickened walls.

Surface of the stem set with divergent fascicles of unbranched, aseptate, or sparingly secondarily septate, hyphae –500×3–5 μ, with pale brown walls 0·5–1 μ thick (browner in the older parts of the stem) and blunt, cylindric, clavate or subcapitate ends, often constricted more or less at one or more points, not encrusted, arising from the superficial generative hyphae and appearing as skeletal hyphae of limited growth: along the main axis, these superficial hyphae becoming shorter upward and appearing as subcylindric

to clavate or subcapitate caulocystidia –80×7–15(–20)μ, with a rather abrupt transition to the hymenium.

I have based this revised description on my Brazilian gatherings. The species is easily recognized from the rather intense brown colour of the main axis and the strigose-tomentose aspect. *P. taxiformis* appears to be allied, as

Fig. 8. *Pterula juruensis*, full-grown fruit-bodies, ×5 (Corner 772).

a reduced state with slender and much less villous axis, slender branches and smaller spores: the superficial hyphae of the stem are less organized in *P. taxiformis* than in *P. juruensis*, which also has caulocystidia in the upper part of the axis and, thus, connects with *P. brunneosetosa*, &c.

P. longispora sp. nov.

Fig. 9.

–2 cm. high, slender, the stem *with a few adventitious branches near the apex: stem* –1·5 cm×0·3 mm., *brownish, finely puberulous, the hairs pale brownish in the lower part* of the stem: *branches* –5 mm.×0·15 mm. wide, filiform, tapered, *simple, white:* horny-tough.

On dead leaves in the forest: Brazil (Rio de Janeiro, Corcovado, *c.* 500 m. alt., Nov. 24, 1948, Corner 773, *type*).

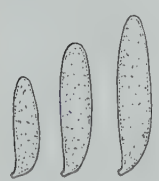

Fig. 9. *Pterula longispora*: spores, ×1,000 (type).

Spores 13–20×3·5–4·5μ, white, smooth, elongate, cylindric-ellipsoid, sub-acute, thin-walled, apparently aguttate, the small apiculus 0·5μ long.

Basidia *c.* 30×10–11μ, clavate: sterigmata 4.

Hymenium not thickening: subhymenium slight: cystidia absent.

Caulocystidia –120×7–12 μ, narrowed rapidly into a subcylindric or filiform appendage-like apex 2–3 μ wide, the walls 0·5–1 μ thick, pale brownish in the lower part of the stem, not encrusted.

Stem-surface sclerotioid with a layer one hypha thick.

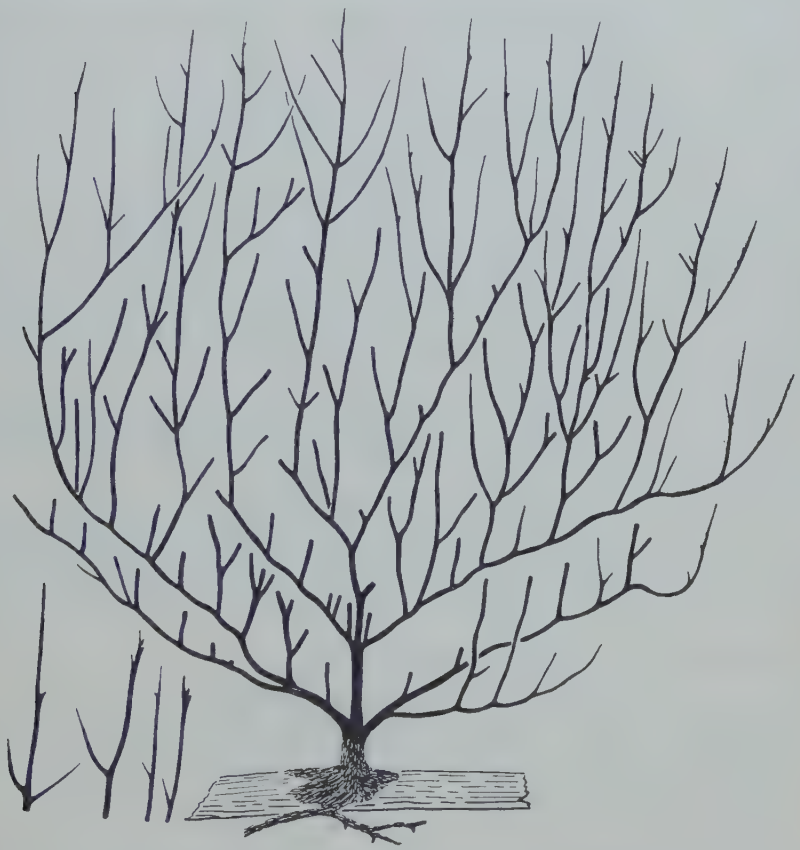

Fig. 10. *Pterula loretensis*, fruit-body, nat. size: branch-tips, ×3 (type).

Hyphae dimitic: skeletal hyphae 2·5–5 μ wide, the walls 0·5–1 μ thick, colourless: generative hyphae 2–4 μ wide, clamped.

This is near to *P. fluminensis* but has longer spores and paler, filiform-appendaged, caulocystidia.

P. loretensis sp. nov.

Fig. 10.

–12 cm. high and wide, *densely caespitose, very bushy, white, then pallid dingy alutaceous or subochraceous, finally somewhat dingy flesh-colour: stem* –20×3–5 mm., *stout*, thickening with age, subvillous at the base, dividing into several,

equal, oblique branches: *branches* 1–1·5 (–2) mm. wide below, *with adventitious, but acropetal, secund branching along the upperside of the oblique branches*, the branchlets 0·2–0·3 mm. wide, *the tips always filiform*, never spathulate and dichotomous: hymenium absent from the uppersides of the branches: fleshy, firm, then tough, with waxy hymenium: *smell strong, fragrant*, somewhat of aniseed.

On dead wood and rubble at the base of rotten trees in the forest: Peru (Iquitos, Apr. 11, 1948, Corner 753).

Spores 4–5 × 2·3–3 μ, white, smooth, pip-shaped, thin-walled, aguttate (or a few small guttulae, when dried), apiculus minute.

Basidia *c.* 20 × 4·5 μ: sterigmata 4.

Hymenium thickening to 70 μ: cystidia absent.

Stem glabrous, sterile hymenium and caulocystidia wanting.

Hyphae dimitic: skeletal hyphae 2·5–4·5 μ wide, the walls 0·7–1·5 μ thick, unlimited, unbranched, aseptate: generative hyphae 2–4·5 μ wide, clamped, in places up to 8 μ wide, thin-walled, sometimes branched from the clamp, or with the clamp inflated 2–4 μ wide: mycelial hyphae 0·7–2 μ wide, both as generative and skeletal hyphae.

These fruit-bodies are the largest yet recorded for the genus: if inflated to the extent found in *Ramaria*, they would reach 30–40 cm. high, which is greater than any known 'Clavaria'.

The secund, adventitious branching and the absence of a distinct axis from the fruit-body, as well as the very small spores, are characteristic: perhaps also the smell. The fruit-bodies were growing in a troop in the small patch of forest on the other side of the river from Iquitos.

P. moniliformis (P. Henn.) comb. nov.

Basinym: *Lachnocladium moniliforme* P. Henn., Hedw. 1904.

Synonyms: *Thelephora clavarioides* Torr., Brot., Ser. Bot. 12, 1914, 61 (*fide* Bresadola).

Hypolyssus clavarioides (Torr.) Lloyd, Lett. 54, 1915, n. 206.

Fig. 11.

In my Monograph I retained this species in *Lachnocladium* because I thought that the swollen joints indicated affinity with true *Lachnocladia* with this 'gouty' character. Examination of the type of *T. clavarioides* (Lloyd Cat. No. 31353, leg. C. Torrend, Bahia, Brazil) shows that it is a *Pterula* with strongly dimitic hyphae, the skeletals being thicker-walled and better marked than in any other species that I have seen, and that it is a very remarkable species. In Torrend's type there are two kinds of fruit-body, the 'gouty', which is sterile, and the normal, and I could see no organic connexion between them, but it seems that the São Paulo specimens, described by Hennings, had the normal, if rather stout, Pteruloid branches growing from the 'gouty' stems. The structure of both fruit-bodies in Torrend's collection was identical and it would be strange for them to be put in the same packet if they were not

observed to be conjoint growths. The species needs further study from careful collections (for the fruit-bodies fragment easily), because it suggests a new element in the genus.

FIG. 11. *Pterula moniliformis*, fragments of moniliform fruit-bodies (below) and a fertile fruit-body with 'pseudopodium' of sand-particles (above), ×2: spores, ×1,000 (Torrend, Bahia).

The following is the description of Torrend's type:

Gouty fruit-bodies –3 cm. high, sterile, *coarsely and moniliformly branched, with more or less pyriform joints* 5–9×3–8 mm. wide above and *with a stalk-like base* 1–2·5 mm. wide, *producing irregular apical whorls of 2–5 similar joints*, often slightly compressed at the apex: drying dull ochraceous or alutaceous with hard, subligneous, paler flesh: hymenium absent: dimitic with very abundant skeletal hyphae.

Pteruloid fruit-bodies –4·5 cm. high, *with the lower part forming a stout*,

fibrilloso-subvillose stem –4 mm. wide, involving many sand-particles, *dividing palmately into rather few, stout branches* 1–2 mm. wide: branches *dilating* –4 mm. wide *at the multifid axils*, rather few, bearing more or less dichotomous coarse branchlets *c.* 1 mm. wide (? branched in one plane): drying fuscous brown and horny.

On the ground: Brazil (Bahia, leg. C. Torrend).

Spores 6–7×3·5 μ, as in *P. plumosa.*

Basidia *c.* 30×7 μ: sterigmata 4.

Hymenium thickening: cystidia none.

Skeletal hyphae 4–6 μ wide, the walls 1–2 μ thick, very abundant and well developed in all parts of both kinds of fruit-body, unbranched, of unlimited growth: generative hyphae 2·5–6 μ wide, thin-walled, clamped, abundant.

P. multifida Fr.

Monogr. Hym. Suec. 2, *1863*, 282: Lloyd, The Genus Pterula, *1919*, f. 1464 (type-specimen).

Synonyms: *P. subulata* auctt. (non Fr.): Corner, Monogr. *1950*, 523.
P. abietis Lloyd, Myc. Notes 75, *1925*, 1257, f. 3200: Corner, Monogr., *1950*, 506.

Fig. 12.

–6 cm. high, *solitary or gregarious*, *drying alutaceous*, pallid ochraceous or brownish drab, often with horny tips: stem –12×0·5–1·5 mm., rather slender: *branches* 0·1–0·5 mm. thick, dilated to 1 mm. at the wider multifid axils, *spreading*, becoming dichotomous with subulate tips, *not anastomosing*.

In coniferous humus and on twigs (*Pinus*, *Picea*, *Abies*): Europe (Sweden, France, Germany, Austria), North Africa (Algeria).

Spores 5·5–7·5×2·7–3·5 (–3·7) μ, white, smooth, more or less pip-shaped to subamygdaliform, the apiculus 0·5–0·7 μ long.

Basidia 27–33×7–8 μ: sterigmata 4, 4 μ long.

Hymenium thickening to 120 μ: cystidia none.

Skeletal hyphae 3–5 (–6) μ wide, the walls 0·5–1 μ thick, as in *P. subulata* but rather better marked: generative hyphae clamped.

Collections examined: Herb. E. Fries (hort. Bot. Uppsal., leg. E. P. Fries, 1853, on Picea-twigs: *type*).

British Museum: Rabenhorst 1512 (Austria, Salzburg, on Abies-needles, det. *P. subulata*): Rabenhorst 2819 (Germany, Württemberg, Schörzingen, 'in pinetis', det. *P. subulata*): Sauter 723 (Austria, Salzburg, det. *P. subulata*): Sydow Myc. Germ. 2459 (Germany, Rhineland, Waldbröl, on Abies-needles, det. *P. subulata*).

Lloyd Cat. No. 32723 (France, Dijon, leg. Barbier, on Picea-needles, det. *P. multifida*): No. 32752 (Algeria, leg. R. Maire, Sept. 16, 1920, on Abies-needles, det. *P. multifida*: *type* of *P. abietis* Lloyd).

Through the kindness of Dr. Lundell I have been able to examine the type

of *P. multifida*, consisting of five small sheets, in the Friesian herbarium at Uppsala: it has spores 6–7·5 × 3–3·5 μ. The species has been confused with *P. subulata* by German mycologists as well as by Lloyd, who then described it as a new species: the French mycologists, however, may have had the correct tradition of *P. multifida*, though latterly confusing it with *P. subulata*. I have noted the differences between them under *P. subulata*, which appears to be the less uncommon. *P. multifida* needs redescription from living specimens: the brief description, copied in my monograph, is erroneous.

FIG. 12. *Pterula multifida*, nat. size: *a*, Rabenhorst 2819; *b*, Rabenhorst 1512; *c*, Sydow 2459; *d*, Sauter 723.

In the British Museum are four collections, referred to *P. multifida*, from Ceylon (Herb. Phillips, Herb. Broome, and Herb. Berk.). They have copious spores 6–7·5 × 3–3·7 μ and skeletal hyphae as in *P. multifida*. At first I thought that they were the Malayan *P. vinacea*, but their fruit-bodies are smaller and have more slender branches, distinctly multifid below. Their habitat must be different from that of *P. multifida*, also, for which reason I think that they may belong to *P. Uleana* or to *P. plumosa* sensu lato.

P. navicula sp. nov.

Fig. 13.

–25 mm. high, slender, subcaespitose, *sparingly branched, the stem and lower parts of the branches pale fuscous or pale fuliginous, the upper parts pallid isabelline whitish*: stem –10 × 0·5 mm., subpuberulous: branches clustered near the stem-apex, simple or once or twice dichotomous, acute, the hymenium absent from the uppersides, subpuberulous: horny-tough.

On more or less buried wood in the forest: Venezuela (near Caracas, *c.* 1800 m. alt., 11 Oct. 1947, Corner 774, *type*).

Spores 16–19 × 7·5–9 μ, white, smooth, the wall 0·5–1 μ thick, the dried spore with contracted oleaginous contents, the apiculus 1 μ long, the apex blunt or subacute, 4–6-angled in optical t.s.

Basidia 55–70 × 14–17 μ, gradually tapered to the base 3–4 μ wide: sterigmata 4.

Hymenium apparently not thickening: subhymenium 15–20 μ thick: the sterile hymenium composed of sterile basidia and incipient cystidia.

Cystidia –80×7–9 μ, frequent or scattered, thin-walled, colourless, smooth, more or less lanceolate with subacute or slightly appendaged apex, projecting –30 μ, arising from the base of the subhymenium.

Stem-surface not truly sclerotioid, with sterile immature basidia and short, subcylindric, blunt, thin-walled, and colourless caulocystidia –50×6–9 μ, arising from a thin subhymenium.

Fig. 13. *Pterula navicula*, spores, basidium, and cystidia, ×1,000 (type).

Hyphae dimitic: skeletal hyphae 3–4·5 (–5) μ wide, the walls 0·5–1 μ thick, the lumen wide, unbranched, unlimited, aseptate: generative hyphae 2–4 μ wide, clamped, with thin or firm walls.

The large spores and basidia of this species are interesting and appear to relate the small-spored species of *Pterula* with *Pterulicium* and *Deflexula*. The species indicates that truly bushy, cystidiate, members of *Pterula* must occur with fruit-bodies as large as in *P. subulata*, but with the thick-walled, navicular or mango-shaped spore.

P. palmicola sp. nov.

Fig. 14.

–5 cm. high, fasciculate, laxly and adventitiously branched: stem 0·3–0·5 mm. wide, arising from a slight superficial mycelium: branches multifid below with slightly expanded axils, tapering to very slender subulate tips, branchlets often more or less secund.

On dead fronds of *Cocos Romanzoffiana*: Brazil (Sta. Catarina, Blumenau, Nov. 1888, Ule 769, det. *P. plumosa* Fr.: type in herb. British Museum).

Spores 9–10·7 × 4·7–6 μ, white, smooth, ellipsoid to subamygdaliform, the walls slightly thickened, the apiculus 1 μ long.

(Hymenium almost entirely disintegrated.)

Hyphae dimitic: skeletal hyphae mostly 2·5–4·5 μ wide, with the walls 0·5–1 μ thick, but in places –12 μ wide: generative hyphae clamped.

Mycelial patch sterile, composed of generative and skeletal hyphae.

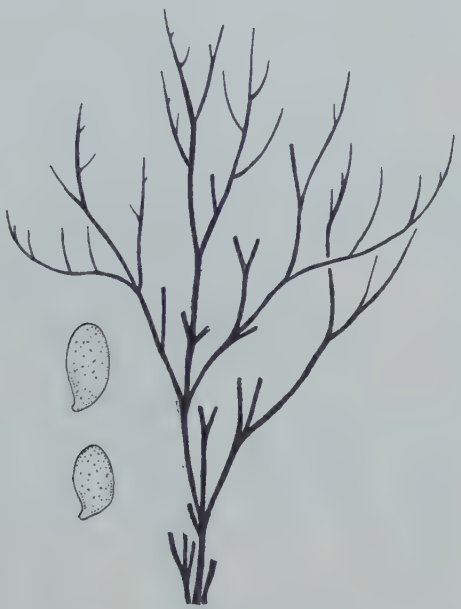

FIG. 14. *Pterula palmicola*, × 2: spores, × 1,000 (type).

The large spores, as in *P. secundiramea* Speg., prevent this from being identified with *P. plumosa*. There is also no indication of the branches being secund, but compare *Clavaria secundiramea* Lév. (on p. 521 of my monograph). *P. capillaris*, according to the Peruvian specimen, has smaller spores, but appears to be similar in other respects.

P. plumosa (Schw.) Fr. *sensu lato*

Synonyms: *P. divaricata* Pk., Rept. N.Y. St. Mus. 32, 1879, 36.

P. penicillata Berk. ex Lloyd, Myc. Writ. 5, 1919, 863, f. 1466, 1467 (ut *P. penicellata*).

Fig. 15.

–4·5 cm. high, *much branched from a distinct stem*, more or less fasciculate, bushy, drying pallid cream to greyish white with the branchlets often fuscous and horny: *stem* –12 × 1–2 mm., glabrous or becoming sparsely strigose,

arising from a mycelial patch with fine fibrillar rhizomorphs: *branches* 0·5–1 mm. wide below, tapering upwards, *polychotomous and multifid below*, with slightly dilated axils, *dichotomous above with slender filiform tips*.

On the ground in dicotyledonous forest, or in gardens: U.S.A., Canada, Japan, South Africa.

FIG. 15. *Pterula plumosa*, fruit-body, ×3: branch-tips, ×6: spores, ×1,000 (Lloyd Cat. No. 5262).

Spores 6–8·7×2·7–3·5 μ, white, smooth, *narrowly ellipsoid to subcylindric*, thin-walled, with small apiculus (*c.* 0·5 μ long).

Basidia *c.* 27×6·5 μ: sterigmata 4.

Hymenium thickening to 120 μ, absent from the stem and the lower parts of the main branches, often unilateral: cystidia none.

Skeletal hyphae 3–6 (–7) μ wide, the walls 0·5–1·2 μ thick, often irregular and kinked, occasionally branched (particularly in the main stem): generative hyphae clamped, often branched from the clamp.

Stem-surface evidently with a slight sterile hymenium.

After much thought and repeated comparison of material, I have decided that all the collections which I list below must be regarded as forms of one

variable species. Part of the variability is no doubt spurious and caused merely by the age of the fruit-bodies, but some forms (*P. plumosa* s. str.) are more slender than those which Lloyd referred, in the main, to his *P. penicillata*. The collections are known almost entirely from the dried state, though Lloyd gives a good photograph of the robust form of his *P. penicillata* in the living condition: the species now requires extensive study in the living state.

The description, given above, is based on the following collections, most of which were referred by Lloyd to *P. penicillata* (though this is a later homonym of *P. penicillata* Fr.):

U.S.A.: Lloyd Cat. Nos. 5262, 14649, 45336 (Minneapolis, Minn., leg. Mary S. Whetstone); 45317 (Peavine Creek, Emma, Lafayette Co., Mo., leg. C. H. Demetrio); 34369, 45318 (Cincinnati, Ohio, leg. Lloyd).

Canada: U.S. Dept. Agr. Myc. Coll. (Vancouver, Fauquier Co., near Thorofare Gap, leg. H. A. Allard, s.n.).

Japan: Lloyd Cat. No. 32742 (Prov. Bungo, leg. A. Yasuda n. 671).

S. Africa: Lloyd Cat. No. 32746 (Pretoria, Fountains Valley, leg. A. M. Bottomley n. 18183).

P. penicillata Berk. ined. (type in herb. Cooke, Kew, from South Carolina, U.S.A., 'inter Asparagos in horto').

Young specimens of these collections are rather divaricate with relatively stout, multifid or polychotomous branches. Old specimens are bushy with more slender, dichotomous branchlets tapering to fine, elongate tips: rarely are there any adventitious branchlets. The type of *P. penicillata* ined. has young fruit-bodies, –2·5 cm. high, with rather more slender stem and main branches than usual. The South African collection agrees fully with the North American, but the Japanese collection is sparingly branched and suggests the link with *P. plumosa* s. str. The spores are identical in all the collections.

P. plumosa s. str.: 2 cm. high, fasciculate or solitary: stem 1 mm. wide, smooth: branches *c.* 0·5 mm. wide below, mostly trifid or irregularly trichotomous below, dichotomous above with fine tips 0·1–0·2 mm. wide, not anastomosing, erect: drying amber-colour, with pallid stem: spores 5·5–6·5 × 3–3·5 μ: hymenium thickening: skeletal hyphae –6 μ wide, rather thin-walled: generative hyphae clamped: on the ground, evidently under dicotyledonous trees, N. Carolina, U.S.A., Schweinitz 1089 (type), in herb. Kew and in herb. Fries, Uppsala.

I have examined both these parts of the type-collection of *P. plumosa*. They appear merely to be young, slender states of the more robust *P. penicillata* sensu Lloyd, though the branches dry a clear amber, rather than fuscous. I can see no specific difference.

Now *P. plumosa* in this wide sense comes very near to *P. multifida* (Europe), *P. Uleana* (Brazil), *P. vinacea* (tr. Asia), and *P. plumosoides* (Brazil).

P. multifida differs in its coniferous habitat and consistently less multifid fruit-bodies, in which respect it resembles *P. plumosa* s. str., and, without a knowledge of the habitat, it would seem impossible to distinguish the dried specimens. Thus, I think that the Ceylon specimens referred to *P. multifida* are *P. plumosa* sensu lato.

P. Uleana, common in Brazil, develops adventitious branchlets and grows to a larger size, with a more slender stem. It has relatively wider spores.

P. vinacea is more slender than *P. plumosa* and is more or less dichotomous throughout. It may also differ in colour and have slightly wider spores.

P. plumosoides differs mainly in habit, having a pronounced stem with strongly divaricate, dichotomous branches, and perhaps also in the colour when living. Nevertheless, the Vancouver collection, which I have placed under *P. plumosa*, has fewer polychotomous branches than usual and its divaricate branches are mainly dichotomous with unilateral hymenium as in *P. plumosoides*.

Fig. 16. *Pterula plumosoides*, ×2 (type).

P. plumosoides sp. nov.

Fig. 16.

–5 cm. high, *caespitose*, *much branched*, *dingy fuscous drab or pallid fuscous flesh-colour*, the hymenium and growing tips whitish: *stem* 1–2·5 cm.×1–2 mm., *rather stout*, sterile, smooth: branches pseudo-polychotomous below (from closely successive dichotomies), clearly *dichotomous* above, not monopodial, spreading, the tips flattened and spathulate before dichotomy, becoming subulate, not cristate: *hymenium unilateral throughout:* tough: smell slight, not unpleasant, rather fungusy.

On humus in the forest: Brazil (Est. do Rio, Saco de São Francisco, Mar. 7, 1948, Corner 770).

Spores 5–7×3–3·7 μ, white, smooth, pip-shaped, thin-walled, with 1–few guttulae when dried, the apiculus *c.* 0·5 μ long.

Basidia 25–32×6·5–7·5 μ: sterigmata 4.

Hymenium thickening to 60 μ: subhymenium *c.* 10 μ thick: cystidia absent.

Stem and bases of the main branches glabrous, without cystidia or sterile hymenium, but the longitudinal hyphae with brownish walls.

Skeletal hyphae 2·5–6 μ wide, of uneven width, the walls 0·5–1 μ thick: generative hyphae 2–6 μ wide, clamped, abundant.

This species is close to *P. plumosa*, but the form of the fruit-body is different, particularly in the conspicuous dichotomous branching, and the colour of the living fruit-body may be different.

FIG. 17. *Pterula secundiramea*, ×2: spores, ×1,000 (type).

P. secundiramea Speg.

Corner, Monograph *1950*, 521.

Fig. 17.

–3 cm. high, *caespitose*, *slender*, *monopodial*, *with secund adventitious*, *simple branches* –1 cm. long, rarely the axis pseudo-dichotomous with one branch as well developed as the main axis, *pale rufescent or flesh colour*, *fuscous downward*: stem 1–10×0·3–0·4 mm., the extreme base sterile and often minutely substrigose with spicular hyphal bundles: branches rather lax, scattered or clustered, filiform, subulate, curved, rarely with an adventitious branchlet.

On rotting branches in the forest: Brazil (Apiahy, Puiggari n. 1487, April–May 1881, Inst. Speg. n. 19269).

Spores 9·5–11×5·5–6·5 μ, white, smooth, amygdaliform, thin-walled, apiculus conspicuous.

Basidia 30–35×10–11 μ: cystidia none: hymenium not thickening.

Skeletal hyphae 3–5 μ wide, unbranched, the walls 0·5–1·5 μ thick: generative hyphae 3–5 μ wide, clamped.

The type-collection is in good condition and has many fruit-bodies showing the characteristic structure of the genus. The individual fruit-bodies are

slender, and from the curvature of the axis and branches they would appear to be geotropic or phototropic. The spores are identical with those of *P. capillaris* and *P. palmicola*. With *P. loretensis*, they appear to be a closely allied group, though the spores of *P. loretensis* are much smaller. (? *Deflexula*.)

P. squarrosa P. Henn.

I have examined the fragment of the type in Bresadola's herbarium (U.S. Dept. of Agriculture Mycological Collections) and find that it is an Aphelaria, as will be reported in a later paper.

FIG. 18. *Pterula stipata*, ×2: spores, ×1,000 (type).

P. stipata sp. nov.

Fig. 18.

–4·5 cm. high, *solitary or gregarious*, not caespitose, *monopodial, with adventitious, mostly simple branches*, drying dark fuscous brown and horny with paler tips: stem –12×0·3–0·7 mm., distinct, glabrous, without hymenium, attached by a very thin, mycelial disc 1–2 mm. wide: branches adventitious, mostly simple, often subopposite, curved ascending, filiform-attenuate, the lower branches on the stouter fruit-bodies usually becoming monaxial with adventitious branches repeating the form of the main axis.

On dead twigs: Brazil (? São Leopoldo, Rio Grande do Sul, leg. J. Rick s.n., Lloyd Cat. No. 30221).

Spores 7–9×4–5 μ, white, smooth, pruniform, thin-walled, with rather prominent, blunt apiculus *c.* 1 μ long.

Basidia *c.* 23×7 μ: sterigmata 4.

Hymenium thickening to 70 μ, absent from the lower part of the main axis and from the bases of the main branches: cystidia none.

Skeletal hyphae –4·5 μ wide, the walls –0·7 μ thick, often ill-formed: generative hyphae clamped: the superficial hyphae of the stem with brownish, agglutinated walls, but not sclerotioid, often secondarily septate: no caulocystidia.

This species has the habit of *Parapterulicium subarbusculum*. I am unable to fit it with any other species of *Pterula*, but compare *P. capillaris*. *P. taxiformis*, with smaller spores and more slender fruit-body, may be allied.

P. subulata Fr.

Linnea 5, *1830*, 531: Lloyd, The Genus Pterula, *1919*, f. 1463 (*type*).

Synonyms: *P. multifida* auctt. (non Fr.): Corner, Monogr. *1950*, 519: Lloyd, The Genus Pterula, *1919*, f. 1465 (= Romell 12053).

Clavaria propera Bourd., Bull. Soc. Myc. Fr. 48, *1932*, 208.

Clavulinopsis propera (Bourd.) Corner, Monogr. *1950*, 384.

Fig. 19.

–6 cm. high, *densely caespitose*, *very bushy*, *pale avellaneous or pale wood brown*, *often tinged flesh-colour below the white tips*, *becoming fuscous from below upwards*, drying horny and fuscous drab: *stem* –10×1–3 mm., *sunk in the ground*, flattened upward: *branches* 1 mm. wide below, more or less flattened, *multifid*, the flattened axils 1·5–2·5 mm. wide, the *branchlets* 0·5–1 mm. wide, *often fimbriate* at the tip, *ascending*, *strict*, *fastigiate*, the old branchlets elongate and subulate, *often concrescent or proliferating* new adventitious branches: waxy-firm, then pliant and rather tough, drying horny and fuscous-rufescent: smell slight, mouldy.

On the ground in woods, parks and flower-beds: Europe, U.S.A.

Spores (5–)5·5–7·5(–8)×(3–)3·5–4·7 μ, white, smooth, rather pip-shaped, thin-walled, aguttate, the apiculus *c.* 0·5 μ long.

Basidia 35–45(–55)×7·5–9 μ, markedly clavate: sterigmata 4, 5–5·5 μ long.

Hymenium thickening to 170 μ, sterile on the upperside of oblique branches and on the stem (as a loose, undeveloped, incipient hymenium): subhymenium *c.* 25 μ thick: cystidia absent.

Hyphae dimitic, but with many intermediates, and the skeletal hyphae often poorly marked: skeletal hyphae 4–7·5 μ wide, long-celled (400–1000 μ), the walls 0·3–1 μ thick, usually as segments of generative hyphae: generative hyphae 3–7·5 μ wide, clamped, shorter-celled, more frequently branched, often branching from the clamps, some with oleaginous contents: hyphal ends at the growing tips 3–5 μ wide: caulocystidia absent.

Collections examined: Herb. Fries ('Gallia: Guepin': *type*).

Romell 12053 (Stockholm, Humlegården, Aug. 11, 1895, on bare soil in the park: issued as *P. multifida* in Fungi Exsicc. Suec. n. 169).

Corner 755 (England, Cambridge, University Botanic Garden, July 31, 1950: issued as *P. multifida*).

Lloyd Cat. No. 32735 (det. *P. multifida*, probably the same collection as Romell 12053).

FIG. 19. *Pterula subulata*, fruit-body, × nat. size: branch, × 3: branch-tips, × 5 (Corner 755).

Lloyd Cat. No. 42093 (U.S.A., Washington, D.C.).

U.S. Dept. Agr. Myc. Coll. (Herb. C. L. Shear: U.S.A., Nebraska, Minden, leg. F. E. Clements, May 27, 1902, under cottonwood logs).

British Museum: Welwitsch 32 (Crypt. Lusit.): Caldesi 973 (Italy): Mougeot et Nestler 995 (France).

This revised description is drawn from my collection 755 of living specimens. In my monograph I based the description of *P. multifida* mainly on Romell 12053 and the description of Konrad and Maublanc, which I now

know should have been referred to *P. subulata*. The two species have been so confused that all previous records must be checked.

Through the kindness of Dr. Lundell I have been able to examine the Friesian types of *P. subulata* and *P. multifida*, from which it is clear that *P. subulata* of German mycologists is really *P. multifida*, and *P. multifida* of recent mycologists is really *P. subulata*. Descriptions of both read much alike, especially as both have the peculiar multifid branching, but it is usually possible to distinguish dried specimens at first glance. The differences are as follows:

P. subulata	*P. multifida*
On bare soil (? also in humus of deciduous forests).	On coniferous twigs and humus.
Stems 1–3 mm. thick, immersed, caespitose.	Stems 0·5–1·5 mm. thick, solitary or gregarious.
Branches 1–2·5 mm. thick below, fastigiate, anastomosing, much divided.	Branches less than 1 mm. wide, slender elongate, not anastomosing.
Drying dark fuscous rufescent; horny: hymenium pallid.	Drying alutaceous, pale ochraceous or pale brownish drab, the tips usually horny.
Spores 3·5–4·7 μ wide.	Spores 2·7–3·5 μ wide.
Skeletal hyphae often rather thin-walled.	Skeletal hyphae well-marked.

Both species have flattened multifid branching. The apex of a branch becomes spathulate and then breaks up into three to many minute growing points, of which two to seven may develop into branches in one plane. The lower branchings are nearly always multifid and the final branchings apparently dichotomous: the change occurs earlier in the slender *P. multifida*.

Both species are dimitic in that distinctly thick-walled, long, and almost unbranched skeletal hyphae occur, but it seems that in all cases they are intercalary segments of generative hyphae, not self-continuing skeletal hyphae. However, as remarked in my monograph, the dimity of *Pterula* is characteristically incomplete. These two European species thus show what may be the beginnings of the dimitic state, as one finds also in many polypores. In *P. subulata* the skeletal hyphae may not become thick-walled until the branches are well developed (for which reason I overlooked them in the type of *Clavaria propera*, as I examined only its side-branches).

The Friesian type-specimen is labelled in Fries's handwriting and so closely resembles his rather artificial illustration that there can be no doubt of its identity. The colour, the anastomosing fastigiate branches, the spores ($6–8 \times 3·5–4·7\ \mu$), and the hyphae agree with those of the other collections which I accordingly refer above to *P. subulata*.

Clavaria propera puzzled me at the time of writing my monograph, for it had not the inflated hyphae or the spores of *Clavulinopsis*, to which I was obliged to refer it. As soon as I saw the living specimens of *P. subulata* in 1950, however, I recognized *C. propera*, and understood how it had been possible to mistake this *Pterula*: its fruit-bodies are unusually stout and multifid for the genus.

P. taxiformis Mont.

Synonym: *P. humilis* Speg. (ined.): Corner, Monogr. 1950, 535 (haud var. *tucumanensis* Speg. = *P. Uleana* P. Henn.).

Fig. 20.

–2 cm. high, scattered or subfasciculate, *slender, monopodial, with few to rather many, adventitious branches, white, then the stem and lower part of the axis brownish fuscous, the hymenium greyish*: stem –5×0·3–0·5 mm., short,

Fig. 20

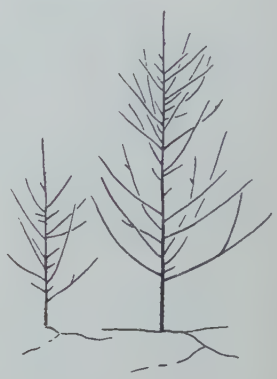

Fig. 21

Fig. 20. *Pterula taxiformis*, ×5: spores, ×1,000 (Lloyd Cat. No. 30222).
Fig. 21. *Pterula taxiformis* var. *gracilis*, ×2 (type).

distinct, *more or less piloso-fasciculate or fibrillose*: branches 0·1–0·3 mm. wide below, tapering to fine tips 10–25 μ wide, simple or occasionally branched with a few adventitious branchlets, or the main axis dividing into 2–3 similar axes with simple branchlets.

On dead sticks, bark and wood in the forest: *French Guiana* (Leprieur 908, type-collection in herb. Bresadola, U.S. Dept. Agr. Myc. Coll.); *Brazil* (Lloyd Cat. No. 30222, 30224, leg. J. Rick, ? Rio Grande do Sul); *Paraguay* (Balansa 2808, 'troncs des arbres morts', Guarapi, Mar. 28, 1880, in herb. Speg. n. 19267, La Plata, as the type of *P. humilis*: Balansa 3348, in herb. Paris, as co-type of *P. humilis*).

Spores 5–7×3·5–5 μ, white, smooth, thin-walled, pip-shaped or lachrymiform, with rather prominent apiculus –1 μ long.

Basidia *c.* 15–20×6–7 μ (? longer): sterigmata 4.

Hymenium apparently not thickening, absent from the stem: cystidia none.

Skeletal hyphae 1·5–3·5 (–4) μ wide in the axis and main branches, the walls –0·7 μ thick, unlimited; 3–6 μ wide in the stem, or inflated –10 μ wide in places, the walls –1·5 μ thick: generative hyphae 1·5–3·5 (–4·5) μ wide, thin-walled, clamped.

Surface of the stem not sclerotioid, but the superficial hyphae with brownish, subagglutinated walls, and with divergent brownish skeletal hyphal ends 5–8 μ

wide, simple or subclavate, loose or somewhat aggregated into fascicles, rarely branched or with 1–2 secondary septa: no caulocystidia.

var. **gracilis** var. nov.

Fig. 21.

Very slender: stem 0·1–0·15 mm. wide, minutely and appressedly piloso-fibrillose, inserted in the wood or with very slender rhizomorphs: branches 0·05–0·12 mm. wide, mostly simple: spores 4·5–6·5 × 3–3·7 μ.

Panama (Upper Rio Chiriqui Viejo, *c.* 1700 m. alt., July 6, 1935, G. W. Martin 2585): *Colombia* (Sierra Nevada de Santa Marta, Dos Aguas, Dept. Magdalena, Aug. 14, 1935, G. W. Martin 3366): *Brazil* (Belem-do-Pará, Sept. 1948, Corner 783): *Peru* (Iquitos, Apr. 10, 1948, Corner 784, ut *typus*).

Typical *P. taxiformis* comes very near to *P. juruensis*, which is more robust and more richly coloured, but intermediate collections may be expected. Var. *gracilis* has not only more slender fruit-bodies, but less fasciculate fibrils on the stem, which may even be merely finely fibrillose, and slightly smaller spores. It seems, also, that the fruit-bodies of var. *gracilis* may degenerate into simple fasciculate clusters. Thus, my Brazilian and Peruvian collections of var. *gracilis* had well-developed fruit-bodies, but Martin's collection from Panama had very small fruit-bodies only 8 mm. high, though similarly branched, and his Colombian collection consisted of simple, or sparingly branched, filiform, clustered fruit-bodies barely 6 mm. high: except for slight spore-differences, as listed below, all these collections of var. *gracilis* were identical microscopically, the brownish, divergent skeletal hyphal ends on the stem being especially characteristic.

In my collections of var. *gracilis* the fruit-bodies appeared to have very slender rhizomorphs: actually, they were very slender rootlets of some flowering plant which were dead, but permeated by the hyphae of the *Pterula*. Martin's collections, however, had true, if slender, rhizomorphs.

The following are the spore-data for *P. taxiformis*:

Balansa 2808	5·5–7 × 4–5 μ	type of '*P. humilis*'
Leprieur 908	5·5–6·5 × 3·5–4 μ	type of *P. taxiformis*
Lloyd 30222, 30224	5–6·5 × 3·5–4 μ	—
Corner (Brazil)	4·5–5·5 × 3–3·5 μ	var. *gracilis*
,, (Peru)	,, ,,	,,
Martin 2585	4·5–6·5 × 3·3–3·7 μ	,,
,, 3366	,, ,,	,,

P. tenuissima (Curt.) Corner

Fig. 22.

The following Brazilian collection seems to belong to this species, though the typical *P. tenuissima* has a longer sclerotioid stem and rather thicker fruit-body. The Brazilian collection has also slightly larger spores, but the difference does not seem material in view of the few spores in the exsiccati

mentioned in my monograph. Possibly the Brazilian collection represents young specimens.

–10 mm. high, 0·1–0·15 mm. wide below, simple, gregarious, filiform-attenuate, the apex 30–50 μ wide, white, the extreme base sterile and brownish.

Fig. 22. *Pterula tenuissima*, spores, × 1,000 (Corner 775).

On dead leaves in the forest: Brazil (Rio de Janeiro, Grajáu, Dec. 18, 1948, Corner 775).

Spores 10–15 × 4–5·5 μ, white, smooth, narrowly amygdaliform, 4–6-angled in optical t.s., the wall distinctly thickened, aguttate.

Basidia 30–35 × 10–11 μ: sterigmata 4.

Hymenium not thickening, eventually fertile over the apex, absent only from the extreme base of the fruit-body: cystidia none.

Base of fruit-body, for a distance of *c.* 50 μ, with a sclerotioid surface: caulocystidia absent.

Hyphae dimitic: skeletal hyphae 2–5 μ wide, the walls 0·5–1 μ thick: generative hyphae 2–5 μ wide, with thin or more or less thickened walls, clamped, often branched from the clamp.

P. Uleana P. Henn.

Synonym: *Pterula humilis* var. *tucumanensis* Speg., An. Mus. Nac. Buen. Air. 19, 1909, 280: Corner, Monograph 1950, 535.

Figs. 23, 24.

–6 cm. high, *bushy*, much branched, *pale ochraceous flesh-colour* with white tips, drying pallid cream with horny branchlets or wholly dark horny, more or less fasciculate: stem –15 × 0·5–1·5 mm. glabrous: branches 0·5 mm. wide below, 0·1–0·3 mm. above, *multifid below, becoming dichotomous above and then developing adventitious branchlets*, the lower branches often elongate and fasciculate, the branchlets often becoming more or less denticulate or subcristate: rather tough, without smell.

On rotten wood or bark, ? also in humus, in the forest: *Brazil* (Sta. Catarina, leg. Ule, *type*-fragment in Brit. Museum: Rio Grande do Sul, leg. J. Rick, Lloyd Cat. Nos. 30220, 32719, 32740 A, 32745, 42082, 42108, 54987; Rick s.n. in herb. Bresadola; Rick 180, 256, and 378 in Mus. Nat. Rio de Janeiro; Herb. Weir, in U.S. Dept. Agr. Myc. Coll., 20579, 20586: Rio de Janeiro, Corner 785, Corcovado, *c.* 500 m. alt.; Bahia, leg. C. Torrend, Lloyd Cat. No. 32736): *Argentina* (Tucuman, Parque Roca, leg. Speg., Inst. Speg. No. 19268, *type* of *P. humilis* var. *tucumanensis*).

Spores 4·5–7 × 2·5–3·5 μ, white, smooth, elliptic to pip-shaped, the wall thin or slightly thickened, the apiculus 0·5–0·7 μ long, apparently aguttate.

Basidia 20–27 × 5–6 μ: sterigmata 4, 3–4 μ long.

Hymenium thickening to 120 μ, unilateral on the lower branches: cystidia none.

Skeletal hyphae 2–5 μ wide, –8 μ wide in places, often of very uneven width, the walls 0·5–1·5 μ thick: generative hyphae 2–5 μ wide, clamped, often branched from the clamp.

Fig. 23. *Pterula Uleana*, × 3: spores, × 1,000: *a*, var. *gracillima* (type): *b*, Herb. Weir 20579; *c*, Rick 378.

var. **gracillima** var. nov.

Fig. 23.

–15 mm. high, very slender, sparingly branched, multifid below, bifid above, with a few adventitious branchlets, drying pallid flesh-drab: stem 0·2–0·3 mm. thick, glabrous.

On dead leaves: Brazil (Bahia, leg. C. Torrend, Lloyd Cat. No. 57712).

Spores 6–7 × 3–3·5 μ: ? hymenium not thickening.

This is evidently a common tropical American species, very variable in size and branching of the fruit-body. It is very near *P. plumosa*, but has less regular and, finally, adventitious branching, a more slender stem, and slightly

shorter, less cylindric spores: it may also grow larger and, according to my collection from Rio de Janeiro, the colour may be different. However, Lloyd 32740 A seems identical in the dried state with *P. plumosa*, and I have little doubt that there will prove to be only one variable and wide-ranging species in the New World, namely *P. plumosa* sensu lato.

FIG. 24. *Pterula Uleana*, branch-tips, ×6; *a*, Lloyd Cat. No. 32736, fruit-body (×3) and branch-tips; *b*, Lloyd Cat. No. 32719; *c*, Lloyd Cat. No. 42082; *d*, Lloyd Cat. No. 32736.

I have examined the fragment of the type-collection in the British Museum. It has abundant true spores among many mould spores, which seem to be the basis of Hennings's spore-measurements. Rick 180 was named *P. pusilla* Bres., the description of which is so different that there would appear to have been some error in labelling: similarly with Rick 256 and 378, named *Lachnocladium Moelleri* (which is *Scytinopogon angulisporus*). The type of *P. humilis* var. *tucumanensis* represents young, or small, fruit-bodies (–25 mm. high) with incipient hymenium: it has few spores 5–5·5×3 μ: the stem is glabrous and the fruit-body is not monaxial, so that the resemblance with *P. humilis* (*P. taxiformis*) is superficial.

Most of the 18 collections, cited above, have spores 4·5–7×2·5–3·5 μ, but Rick 280 and Corner 785 have smaller spores 4·3–5·5×2·7–3·5 μ: Lloyd Cat. No. 32736 has spores 4·7–6·3×3–3·5 μ.

P. velutipes sp. nov.

Fig. 25.

–4·5 cm. high, *fasciculate*, upcurved, sparingly to rather abundantly branched, *multifid below*, *dichotomous above*, drying fuscous brown and horny: *stem* 1–3×0·5–0·8 mm., free or several from a common trunk –3×1 mm., *finely brownish puberulous*, *or velutinate*, *in the lower part*, fertile above, apparently without superficial mycelium: branches slender, fine, lax.

Fig. 25. *Pterula velutipes*, ×3: spores, ×1,000 (Lloyd Cat. No. 42117).

On dead wood: Ecuador (Lloyd Cat. Nos. 42094, 42117 as type, leg. N. A. Nunez; No. 32726, leg. Louis Mille).

Spores 7–9·5×4–6 μ, white, *very slightly angled in optical t.s.*, the wall slightly thickened, ellipsoid, blunt or subacute, with prominent apiculus –1·5 μ long.

Hymenium thickening: cystidia none.

Skeletal hyphae 3–5 μ wide, the walls –1 μ thick: generative hyphae clamped.

Caulocystidia –120×9–17 μ, cylindric subventricose, the base deeply immersed in the 'subhymenium', the apex blunt to subacute, or the apex elongate and acute, aseptate or rarely with a secondary septum: walls 0·5–1 μ thick, the inner layer firm and *brownish*, the thin outer layer colourless and submucilaginous.

Surface of the stem sclerotioid, with a pseudoparenchymatous cortex 25–50 μ thick, with the immersed bases of the caulocystidia; the superficial cells irregular, agglutinated, with brownish walls, often subclavate as an incipient hymenium.

This very distinct species bears out the suggestion, made in my monograph, that there may be cystidate series of the genus. It has the branched fruit-body

combined with the sclerotioid stem-surface and caulocystidia, but lacks hymenial cystidia. It seems related to *P. navicula*. It differs, however, from most other species with caulocystidia in having them immersed in the incipient sterile hymenium on the stem, as in *P. fluminensis*.

P. vinacea Corner

Lloyd Cat. No. 32741 (det. *P. penicellata* Lloyd): 6 cm. high, flesh-coloured: spores 6·5–8 × 3·3–4 μ: on rotting leaves of *Juglans regia*, India (Himalayan range, Kumoun, Almara, leg. S. D. Joshi, Sept. 3, 1921, at the end of the rainy season).

This agrees exactly with the Malayan specimens, but for the slightly wider spores.

PTERULICIUM Corner

P. xylogenum (B. et Br.) Corner

The following collections belong to this species:

Lloyd Cat. Nos. 14114, 14115, 14116, 32738 (det. *P. taxiformis*), 32739 (det. *P. capillaris*), 32754, 37257, 37258: all from the Philippine Islands, on dead bamboo, except 32739 on dead wood.

Lloyd Cat. No. 32737 (on dead sugar-cane, Tela, Honduras): this is the only collection known from the New World, and it may have been introduced.

The larger fruit-bodies in these collections have multifid lower branches and adventitious upper branches. The resupinate corticioid patch was present in all. The spores resemble those of *Deflexula*, but they are not angled in optical t.s.

As noted under *Pterula capillaris*, Lloyd seems to have confused these two species, though there is yet no knowledge as to what was the Javanese type of *P. capillaris*.

NOVITATES PTERULAE

P. brunneosetosa sp. nov.

Receptacula ad 5 cm. alta, simplicia vel paulum ramosa, raro apice penicillato-ramosa, alba vel cremea, dein subalutacea: stipite ad 12 × 0·5–1 mm., pallide cervino, dein fuscocervino, sub lente brunneopiloso: ramis adventitiis, 0·5–1 mm. latis, subulato-filiformibus: sporis 11–14 × 4–5 μ, subamygdaliformibus vel subfusiformibus, tunica distincte incrassata, aguttatis: cystidiis deficientibus: hymenio haud incrassato: caulocystidiis ad 150 × 8–20 μ, conicis, tunicis brunneolis 0·5–1·5 μ crassis: stipitis superficie sclerotioideo, hypha unica stratosa: hyphis skeletalibus 4–6 μ latis: hyphis tenuiter tunicatis septato-fibulatis: ad folia emortua: Brazil (Corner 776, Estado do Rio, Niteroi; Corner 777, Rio de Janeiro): Bolivia (Corner 778 et s.n., Cobija).

P. complanata sp. nov.

Receptacula ad 4 cm. alta, caespitosa, ramis ramulisque complanatis, stipite laevi, axillis flabelliformibus, ramulis dein elongatis et filiformibus: sporis 6–7 × 3–3·5 μ,

tunica vix incrassata: hyphis skeletalibus 2·5–5 μ latis: cystidiis deficientibus: ad terram, Brazil (leg. Gardner, pr. Maranhao, s.n., 1841: typus in herb. Kew.).

P. cystidiata sp. nov.

Receptacula ad 15 × 0·2–0·4 mm., simplicia, raro ramis 1–3 brevibus adventitiis praedita, filiformia, alba dein cremea, stipite brevissimo fusco brunneolo inserta: sporis 10–12·5 × 4·5–5·2 μ, tenue tunicatis, aguttatis, apiculo 1–1·5 μ longo: cystidiis 25–70 × 9–15 μ, tunicis 1–3 μ crassis, apicibus incrustatis, hyalinis, subcylindricis, subventricosis vel clavatis: hymenio haud incrassato: stipitis superficie basi imo sclerotioideo: hyphis skeletalibus 2·5–5 μ latis: hyphis tenuiter tunicatis septato-fibulatis: ad folia emortua: Brazil (Corner 768, Estado do Rio, Niteroi).

P. epiphylloides sp. nov.

Receptacula ad 20 × 0·1–0·2 mm., simplicia, filiformia, alba, stipite sterili (ad 8 mm. longo) fuscobrunneolo: sporis 8–11·5 × 3·5–4·5 μ, subcylindricis, apice subacutis: cystidiis ad 30 × 7–10 μ, tenuiter tunicatis, clavatis vel subcylindricis, hyalinis, haud incrustatis: hymenio haud incrassato: caulocystidiis ad 50 × 7–15 μ, clavatis vel conico-elongatis et basim versus ventricosis, tunicis brunneis et vix incrassatis: stipitis superficie sclerotioideo, hyphis 1–2 stratoso; hyphis skeletalibus 2·5–5 (–8) μ latis: hyphis tenuiter tunicatis septatofibulatis: ad folia emortua: Panama (G. W. Martin 3146, Ins. Barro Colorado, *typus*; G. W. Martin 6194, pr. Fort Sherman).

P. fluminensis sp. nov.

Receptacula ad 3 cm. alta, monopodialia, ramis paucis vel numerosis adventitiis, alba: stipite ad 16 × 0·2–0·4 mm., fusco-cervino, minute brunneo-puberulo, inserto: ramis ad 11 × 0·1–0·2 mm., simplicibus vel ramulis 1–2 praeditis: sporis 7–8 × 3–3·7 μ, anguste ellipsoideis, aguttatis: cystidiis deficientibus: hymenio haud incrassato: caulocystidiis ad 120 × 7–12 μ, conicis, subacutis vel acutis, tunicis brunneolis 0·5–1 μ crassis: stipitis superficie sclerotioideo, 2–4 hyphis stratoso: hyphis skeletalibus 2·5–4·5 μ latis: hyphis tenuiter tunicatis septato-fibulatis: ad folia emortua: Brazil (Corner 771, *typus*, et Corner s.n., Estado do Rio, Niteroi). *P. brunneosetosae* affinis.

P. longispora sp. nov.

Receptacula ad 20 cm. alta, gracilia, alba: stipite ad 15 × 0·3 mm., brunneolo, puberulo, pilis brevibus brunneolis: ramis paucis, adventitiis, ad 5 × 0·15 mm., filiformibus, simplicibus: sporis 13–20 × 3·5–4·5 μ, elongatis, cylindrico-ellipsoideis, tenuiter tunicatis, apiculo parvo 0·5 μ longo: cystidiis deficientibus: hymenio haud incrassato: caulocystidiis ad 120 × 7–12 μ, apicibus elongato-filiformibus 2–3 μ latis, tunicis 0·5–1 μ crassis, brunneolis: stipitis superficie sclerotioideo, hypha unica stratoso: hyphis skeletalibus 2·5–5 μ latis: hyphis tenuiter tunicatis septato-fibulatis: ad folia emortua: Brazil (Corner 773, Rio de Janeiro, Corcovado).

P. loretensis sp. nov.

Receptacula ad 12 cm. alta, magna, caespitosa, ramosissima, alba, dein subalutacea v. subochracea v. subincarnata: stipite ad 20 × 3–5 mm., crasso, basi subvilloso: ramis multifidis: ramulis 0·2–0·5 mm. latis, copiosis, adventitiis, plerumque secundum latus superum ramorum obliquorum, apicibus semper filiformibus: odore fragranti: sporis 4–5 × 2·3–3 μ: hymenio incrassato: cystidiis deficientibus: hyphis skeletalibus 2·5–4·5 μ latis: ad lignum emortuum, in silva, Peru (Corner 753, Iquitos, typus in herb. Cantab.).

P. navicula sp. nov.

Receptacula ad 25 mm. alta, subcaespitosa, paulum ramosa, sordide alba, basim versus subfusca vel subfuliginea: stipite ad 10×0·5 mm., subpuberulo; ramis congregatis ad apicem stipitis, simplicibus vel semel vel bis dichotomis, acutis, subpuberulis: sporis 16–19×7·5–9 μ, tunicis 0·5–1 μ crassis: cystidiis ad 80×7–9 μ, tenuiter tunicatis, hyalinis, plus minus lanceolatis, subacutis vel breviter appendiculatis: hymenio haud incrassato: stipitis superficie haud vero sclerotioideo: caulocystidiis ad 50×6–9 μ, subcylindricis, obtusis, tenuiter tunicatis, hyalinis: hyphis skeletalibus 3–4·5 μ latis: hyphis tenuiter tunicatis septato-fibulatis: ad lignum plus minus humatum: Venezuela (Corner 774, pr. Caracas, *c.* 1800 m. alt.).

P. palmicola sp. nov.

Receptacula ad 5 cm. alta, laxe ramosa, ramis adventitiis: stipite 0·3–0·5 mm. lato, mycelio sparso orienti: ramis subtus multifidis, axillis subdilatatis, superne subulatis, ramulis plus minus secundis, siccis subochraceis: sporis 9–10·7×4·7–6 μ, tunicis vix incrassatis, ellipsoideis vel subamygdaliformibus: cystidiis deficientibus: hyphis skeletalibus 2·5–4·5 (–12) μ latis: hyphis tenuiter tunicatis septato-fibulatis: ad folia emortua palmarum (*Cocos Romanzoffiana*): Brazil (Sta. Catarina, Blumenau, Ule 769, in herb. Mus. Brit. *typus*).

P. plumosoides sp. nov.

Receptacula ad 5 cm. alta, caespitosa, multum ramosa, pallide fusco-cinerea, hymenio pallido, apicibus ramulorum albidis: stipite ad 25×1–2 mm., bene evoluto, laevi: ramis ramulisque dichotomis, divaricatis, hymenio ex integro unilaterali: sporis 5–7×3–3·7 μ: hymenio incrassato: cystidiis deficientibus: hyphis skeletalibus 2·5–6 μ: ad terram in silva, Brazil (Corner 770, Mart. 1948, Estado do Rio, Saco de Sao Francisco: typus in herb. Cantab.).

P. stipata sp. nov.

Receptacula ad 4·5 cm. alta, solitaria v. gregaria, monopodialia, ramis adventitiis et plerumque simplicibus, 1–2 inferioribus aliquando evoluto ut axe: stipite ad 12×0·3–0·7 mm., laevi, disco mycelio 1–2 mm. lato affixo: sporis 7–9×4–5 μ, tunica tenui, apiculo prominenti: hymenio incrassato: cystidiis deficientibus: hyphis skeletalibus ad 4·5 μ latis: hyphis stipitis superficialibus brunneo-tunicatis et agglutinatis, sed haud sclerotioidibus: ad ramulos emortuos, Brazil (Lloyd Cat. No. 30221, leg. J. Rick, s.n., Rio Grande do Sul).

P. taxiformis var. *gracilis* var. nov.

Gracillima: stipite 0·1–0·15 mm. lato, minute et appresse piloso-fibrilloso: ramulis 0·05–0·12 mm. latis, plerumque simplicibus: sporis 4·5–6·5×3–3·7 μ: ad lignum et et corticem emortuum, Panama, Colombia, Brazil, Peru (Corner 784, Iquitos, ut est typus in herb. Cantab.).

P. Uleana var. *gracillima* var. nov.

Receptacula ad 15 mm. alta, gracillima, paulum ramosa: stipite 0·2–0·3 mm. lato: sporis 6–7×3–3·5 μ: ad folia emortua in silva, Brazil (Lloyd Cat. No. 57712, leg. C. Torrend, Bahia).

P. velutipes sp. nov.

Receptacula ad 4·5 cm. alta, fasciculata, multifida, dein dichotoma: stipite brevi, ad 3×0·5–0·8 mm., brunneo-puberulo v. velutino: sporis 7–9·5×4–6 μ, vix subangulato in sect. transv., tunica vix incrassata: hymenio incrassato: cystidiis deficientibus: hyphis skeletalibus 3–5 μ latis: caulocystidiis ad 120×9–17 μ, cylindrico-subventricosis, basi immerso, obtusis v. acuminatis, tunica paulum incrassata et brunnea: ad lignum emortuum, Ecuador (Lloyd Cat. Nos. 42094, 42117 ut typus, leg. N. A. Nunez, et No. 32726, leg. Louis Mille).

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A Physiological Study of Embryo Development in *Heracleum sphondylium* L.

II. The Effect of Temperature on After-ripening

BY

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With three Figures in the Text

Abstract

The action of low temperature in the after-ripening of seeds of *Heracleum sphondylium* is to make available the endosperm reserves, without which the embryo is starved at room temperature. There is no development of 'secondary dormancy' in the embryo, since the effect of low temperature in after-ripening is strictly additive, and the total length of low-temperature treatment required for germination is in no way influenced by periods at room temperature before or during after-ripening.

Introduction

In a previous paper (Stokes, 1952) it was shown that embryos of *Heracleum sphondylium* can only attain the development and accumulation of nutrients necessary for germination during a period of 2 to 3 months' low-temperature treatment. At room temperature the embryo grows actively only for a short time and the endosperm reserves are not utilized. While it appears probable that the reserves are in a form unavailable to the embryo, there is also the possibility that, though accessible, they are not utilized because of some inability on the part of the embryo to function normally at room temperature.

Carbon dioxide narcosis and shortage of oxygen within the seed are known to cripple the normal respiratory processes in embryos of other species (Kidd, 1914; Davis, 1930; Ransom, 1935), and the development of such a condition within seeds of *Heracleum sphondylium* would account for the observed falling off and cessation of growth at room temperature.

This paper is an attempt to ascertain whether the cessation of growth at room temperature is due to the development of a local environment inhibitory to embryo growth, or, as appears likely, the embryo is merely starved in the presence of unavailable endosperm reserves.

Procedure

The general procedure followed was that already described (Stokes, 1952). Samples of 40 seeds were used for each treatment and seeds were after-ripened in a refrigerator fluctuating between 2° C. and 5° C.

[Annals of Botany, N.S. Vol. XVI, No. 64, October, 1952.]

Experiment 1. *The effect of development at room temperature on subsequent after-ripening at* 2° *C.*

An experiment was performed in which embryos were allowed to develop at room temperature before the seeds were after-ripened. Sets of imbibed seeds were given from 1 to 18 weeks at 15° before being put at low temperature,

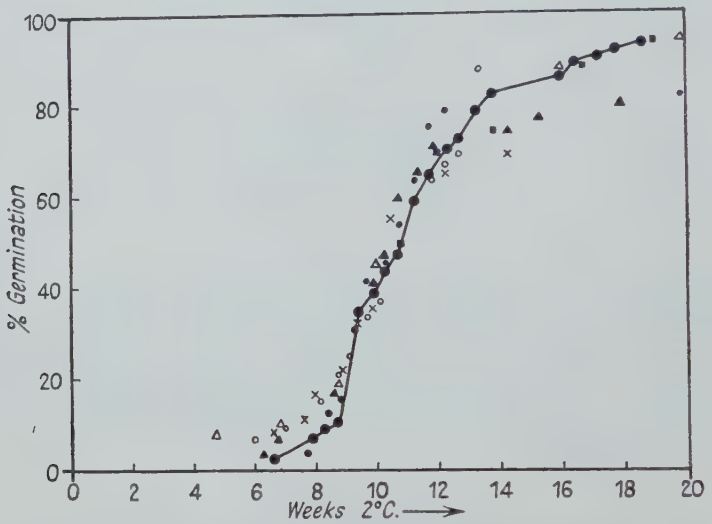

FIG. 1. Length of after-ripening at 2° necessary for germination of seeds which were allowed to develop at room temperature before being put at low temperature.

●—● Control set. No development at room temperature.
▲ 1 week's development at room temperature before after-ripening.
× 2 weeks' " " "
● 4 " " " "
○ 6 " " " "
△ 14 " " " "
■ 18 " " " "

where they were left to germinate. It has been shown that at room temperature growth proceeds to a size half of that reached at the end of after-ripening. There were therefore no very small embryos at the commencement of after-ripening. All were advanced in their development and some were already half the necessary size at the commencement of after-ripening.

Results given in Fig. 1 show that, in spite of the preliminary development at room temperature, the length of after-ripening subsequently required for germination was the same in all cases and not reduced below the requirements of seeds which had no preliminary development.

Experiment 2. *The interruption of low-temperature treatment by periods at room temperature and its effects on the after-ripening process.*

In this experiment seeds were transferred to room temperature for various lengths of time after 6 weeks' after-ripening.

These interruptions were for 4 days, 1 week, 2, 3, 4, 5, and 6 weeks.

The results are given in Fig. 2. Only the germinations on and after the return to the refrigerator are plotted, and the time scale used is the total length of low-temperature treatment given. Thus all curves start at 6 weeks, as all seeds have received that amount of cold when returned to the refrigerator.

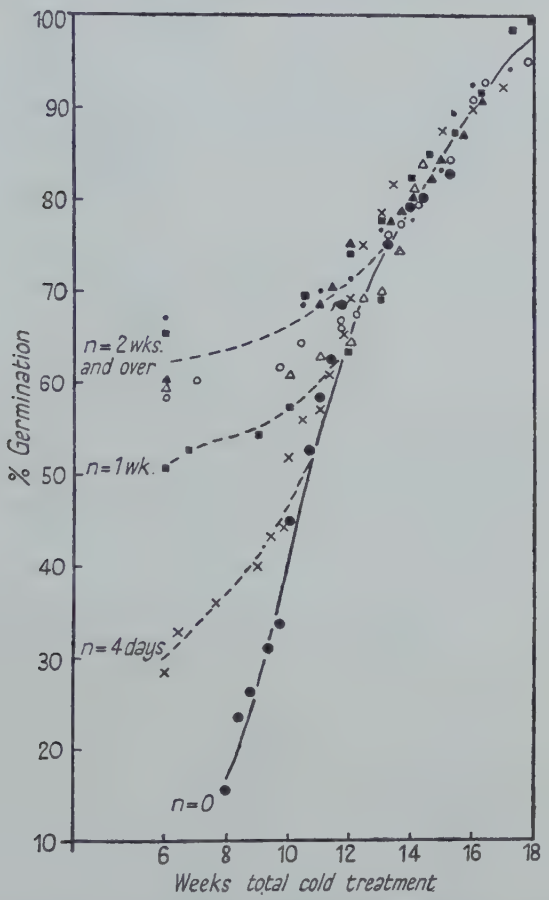

FIG. 2. Graph to show germination of seeds on and after their return to the refrigerator following a period at room temperature given after 6 weeks' after-ripening. *n* denotes the length of the period at room temperature.

The control set had no interruption to after-ripening.

Seed used was that of the 1950 harvest, and it will be seen that this had a lower after-ripening requirement than previous years' seed, since a 50 per cent. germination is obtained after 10 weeks at 2° in comparison with 12 weeks in other years. Also 6 weeks at low temperature was sufficient to complete the after-ripening of 60 per cent. of the seed, since these germinated during the period at room temperature. The lower figures for germination in

the 4 days and 1-week set are because these times were not long enough for all the after-ripened seeds to germinate before they were returned to the refrigerator.

In the sets where all the after-ripened seeds germinated at room temperature (interruption of 2 weeks or over) a period of about 5 weeks elapsed at low

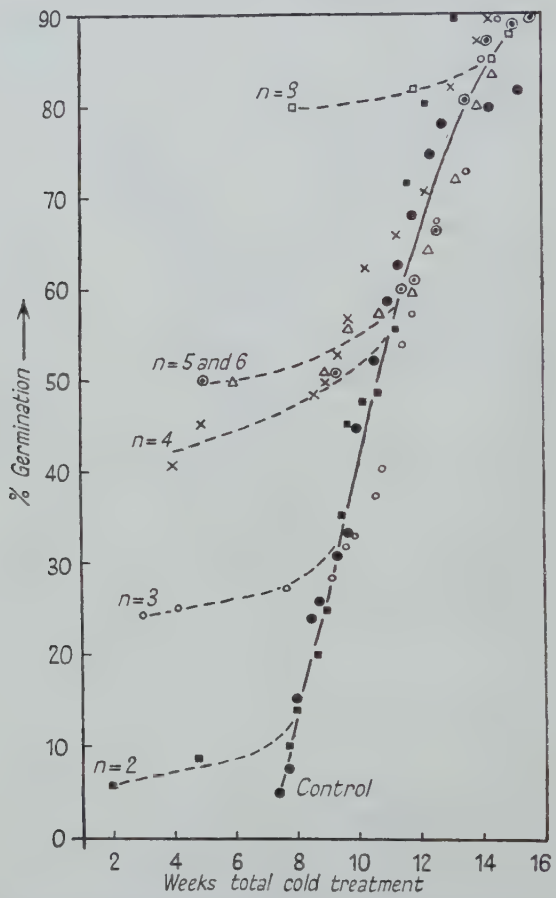

FIG. 3. Graph to show germination of seeds on and after their return to the refrigerator following a period of 4 weeks at room temperature given after 2, 3, 4, 5, 6, and 8 weeks' after-ripening. *n* denotes the number of weeks after-ripening given before the interruption at room temperature.

temperature before any further germinations took place. This is because more after-ripening was required for these seeds, and then, as has been explained previously (Stokes, 1952), this had to be followed by 3 or 4 weeks before the emergence of the radicle at low temperature.

Above the 70 per cent. level, however, these factors do not complicate the results and it is clearly shown that in all groups the curves follow the same course.

The conclusion, therefore, is that the total length of after-ripening required is not influenced by interruptions taking place after 6 weeks.

A further experiment was conducted to verify that this result was applicable at any stage during after-ripening.

Four weeks at room temperature was selected as a suitable period of interruption, and this was given after 2 weeks, 3, 4, 5, 6, and 8 weeks' after-ripening.

The results are plotted in Fig. 3. Again only those germinations on and after the return to the refrigerator are plotted. Here the amount of germination taking place during the interruption indicates the percentage which was after-ripened before removal to room temperature. Thus 25 per cent. germinated in the warmth after 3 weeks' and 45 per cent. after 4 weeks' after-ripening.

The graph shows, in the same way as before, that the effect of low temperature on after-ripening is strictly additive, and the total length of time required at low temperature is the same regardless of how it may be split up or interrupted.

Discussion and Conclusion

The result obtained in expt. 1 does not of itself disprove the existence of some inhibitor to growth. The result given in Fig. 1 could possibly be explained on the hypothesis that during development at room temperature some growth inhibitor is gradually built up until it finally stops growth altogether. The necessity for a period at low temperature, approximating in length to that usually required for development at low temperature, could then be explained on the basis that such a period of low-temperature treatment was necessary for the removal of the inhibitor. Low temperature is, indeed, known to have this action in seeds of *Xanthium* and *Ambrosia trifida* and *Polygonum scandens* (Davis, 1930; Ranson, 1935).

In such a case, however, additional low-temperature treatment would be necessary for germination in some cases described in expt. 2. Let us assume, for example, that n weeks' development at low temperature is normally necessary for germination, and that x weeks' subjection to low temperature are necessary for removal of the inhibitor developed during 4 weeks at room temperature. Then, taking the case of the set in expt. 2 which was given 4 weeks' after-ripening, followed by 4 weeks in the warmth: for germination, a further x weeks at low temperature for the removal of the inhibitor plus n-4 weeks to complete the development would be necessary, making a total of $n+x$ weeks' low-temperature treatment in all. But experiment shows that, in fact, n weeks only are required, since the total length of after-ripening needed is not increased by interruptions at room temperature.

There is therefore no question of room temperature reversing the previous effect of low temperature, or of low temperature being necessary to remove the effects of room temperature.

The simplest explanation of the observations recorded in these experiments

is that suggested in a previous paper (Stokes, 1952), namely, that after-ripening is concerned not with the removal or prevention of an inhibitor to growth itself, but with the transference of food materials from the endosperm. This transference occurs only at low temperature, and since the growth of the embryo is dependent on the supply of nutrients from the endosperm, germination only takes places after a fixed period of time at low temperature.

Summary

1. Development at room temperature prior to after-ripening does not reduce the length of low-temperature treatment necessary for germination.

2. It is shown that there is no development of secondary dormancy at room temperature since the total length of low-temperature treatment necessary to complete after-ripening is strictly additive, and in no way influenced by interruptions at room temperature.

3. The action of low temperature in after-ripening is, therefore, to make available the endosperm reserves without which the embryo is starved at room temperature.

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The post is vacant from February, 1953. The University reserves the right to appoint a person other than one of the applicants or to make no appointment.

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The closing date for the receipt of applications is 30 November 1952.

PHYTOMORPHOLOGY

A Journal of Plant Morphology

CONTENTS OF VOLUME 2, No. 1. PUBLISHED IN MARCH 1952

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